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(54) Title: RECOMBINANT YEASTS FOR EFFECTIVE FERMENTATION OF GLUCOSE AND XYLOSE

(57) Abstract

Described are recombinant yeasts containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase, and DNA molecules, vectors and methods useful for producing such yeasts. The recombinant yeasts effectively ferment xylose to ethanol, and preferred yeasts are capable of simultaneously fermenting glucose and xylose to ethanol thereby taking full advantage of these two sugar sources as they are found in agricultural biomass.

gene integrated at
early or multiple
minutes
site

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RECOMBINANT YEASTS FOR EFFECTIVE FERMENTATION OF GLUCOSE AND XYLOSE

BACKGROUND OF THE INVENTION

The present invention relates generally to
5 genetically engineered yeasts capable of simultaneously
fermenting the two major sugar constituents of cellulosic
biomass, glucose and xylose, to ethanol. More
particularly, the present invention relates to such yeasts
which can be constructed by cloning a xylose reductase
10 gene, a xylitol dehydrogenase gene, and a xylulokinase
gene in yeasts capable of fermenting glucose to ethanol.

Recent studies have proven ethanol to be an ideal
liquid fuel for automobiles. It can be used directly as a
neat fuel (100% ethanol) or as a blend with gasoline at
15 various concentrations.

The use of ethanol to supplement or replace gasoline
can reduce the dependency of many nations on imported
foreign oil and also provide a renewable fuel for
transportation. Furthermore, ethanol has proven a cleaner
20 fuel that releases far less pollutants into the
environment than regular gasoline. For example, it has

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It has been demonstrated that the use of oxygenated materials in gasoline can reduce the emission of carbon monoxide, a harmful pollutant, into the air. Among the several oxygenates currently used for boosting the oxygen content of gasoline, ethanol has the highest oxygen content. The United States Environmental Protection Agency (EPA) has shown that gasoline blended with 10% ethanol reduces carbon monoxide emissions by about 25%-30%.

Up to now, the feedstock used for the production of industrial alcohol by fermentation has been sugars from sugar cane or beets, starch from corn or other food crops. However, these agricultural crops are too expensive to be used as feedstock for the large-scale production of fuel ethanol.

Plant biomass is an attractive feedstock for ethanol-fuel production by fermentation because it is renewable, and available at low cost and in large amounts. The concept of using alcohol produced by microbial fermentation of sugars from agricultural biomass had its nascence at least two decades ago. The major fermentable sugars from cellulosic materials are glucose and xylose (with the ratio of glucose to xylose being approximately 2 or 3 to 1). The most desirable fermentations of cellulosic materials would, of course, completely convert both glucose and xylose to ethanol. Unfortunately, even now there is not a single natural known microorganism capable of fermenting both glucose and xylose effectively.

Yeasts, particularly Saccharomyces, have traditionally been used for fermenting glucose-based materials to ethanol, and they are still the best microorganisms for converting glucose to ethanol.

However, these glucose-fermenting yeasts have been found not only unable to ferment xylose but also unable to use the pentose sugar for growth. Nevertheless, these glucose-fermenting yeasts can use xylulose for growth and fermentation (Figure 1), albeit with varying efficacy. For example, *S. cerevisiae* ferments xylulose very poorly while species of *Schizosaccharomyces* does so quite effectively (Chiang et al., 1981; Lastick et al., 1989).

Even though the glucose-fermenting yeasts are unable to use xylose both for growth and fermentation, there are many natural yeasts that can use xylose for growth aerobically but they cannot ferment xylose to ethanol. These xylose-using/non-fermenting yeasts rely upon two enzymes--xylose reductase and xylitol dehydrogenase--to convert xylose to xylulose. These yeasts are different from most bacteria which rely on a single enzyme--xylose isomerase--to convert xylose directly to xylulose (Figure 1). The yeast xylose reductase and xylitol dehydrogenase also require cofactors for their actions; xylose reductase depends on NADPH as its cofactor and xylitol dehydrogenase depends on NAD as its cofactor. On the contrary, bacterial xylose isomerase requires no cofactor for direct conversion of xylose to xylulose (Figure 1).

Two decades ago, much effort was devoted in an attempt to find new yeasts capable of effectively fermenting both glucose and xylose to ethanol. Although no such ideal yeast has been found, those efforts did have limited success. For example, a few yeasts were found to be capable not only of utilizing xylose for growth aerobically, but also of fermenting xylose to ethanol (Toivola et al., 1984; Dupreer and vander Walt, 1983), although none of these xylose-fermenting yeasts were totally effective in fermenting xylose to ethanol

(Jeffries, 1985). In addition, these yeasts are unable to ferment glucose effectively.

Among the xylose-fermenting yeasts, three species, Pachysolen tannophilus (Toivola et al., 1984), Candida shehatae (Dupreez and van der Walt, 1983), and Pichia stipitis (Grootjen et al., 1990) have been extensively characterized. P. stipitis and C. shehatae ferment xylose better than other xylose-fermenting yeasts (Grootjen et al., 1990). Nevertheless, even the best xylose-fermenting yeasts lack high efficiency in fermenting xylose, and are also highly ineffective in fermenting glucose (Jeffries, 1985).

In the past decade, efforts were also made to genetically modify traditional glucose-fermenting yeasts, particularly S. cerevisiae, by recombinant DNA techniques. Initial efforts were concentrated on cloning a xylose isomerase gene into yeast to render it capable of converting xylose directly to xylulose without dependence on cofactors. However, these efforts have been unsuccessful because the genes encoding various bacterial xylose isomerases are incapable of directing the synthesis of an active enzyme in S. cerevisiae (Rosenfeld et al., 1984; Ho et al., 1983; Sarthy et al., 1987; Wilhelm and Hollenberg, 1984; Amore et al., 1989)).

In the last few years, efforts toward genetically engineering yeasts, particularly S. cerevisiae, to ferment xylose have been focused on cloning genes encoding xylose reductase (Takama et al., 1991; Hallborn et al., 1991; Strasser et al., 1990), xylitol dehydrogenase (Köetter et al., 1990; Hallborn et al., 1990) and xylulokinase (Hallborn et al., 1990; Chang and Ho, 1990; Deng and Ho, 1990). S. cerevisiae and other

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glucose-fermenting yeasts do not contain any detectable xylose reductase or xylitol dehydrogenase activities, but all seem to contain xylulokinase activity. Thus, the glucose-fermenting yeasts can all ferment xylulose, but do so with varying efficacy (Deng and Ho, 1990).

Recently, Köetter et al. (1990), Strasser et al. (1990), and Hallborn et al. (1990; 1991), have cloned both the xylose reductase and the xylitol dehydrogenase gene in S. cerevisiae. However, these genetically engineered yeasts still cannot effectively ferment xylose. For example, these yeasts have been incapable of fermenting more than 2% xylose. In addition, they produce large amounts of xylitol from xylose (Hallborn et al., 1990; Köetter and Ciriacy, 1993), which diverts the valuable xylose substrate from the desired fermentive path to ethanol.

The extensive background in this field as outlined above demonstrates that despite the concerted and longstanding efforts of numerous researchers, yeasts capable of effectively fermenting both glucose and xylose to ethanol have not been achieved. Accordingly, there remain needs for such yeasts and for methods of their preparation and use. It is to these needs that the present invention is addressed.

SUMMARY OF THE INVENTION

A feature of this invention relates to the discovery that new yeast strains capable of effectively fermenting xylose alone or simultaneously with glucose can be created using recombinant DNA and gene cloning techniques. Particularly, these techniques have been used to create new recombinant yeasts containing cloned xylose reductase (XR), xylitol dehydrogenase (XD), and xylulokinase (XK) genes which are fused to promoters not inhibited by the presence of glucose.

Accordingly, one preferred embodiment of the invention provides a recombinant yeast strain containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and capable of fermenting xylose to ethanol. The recombinant yeast strain is preferably also capable of fermenting glucose to ethanol, and more preferred such yeast strains which can effectively ferment these two sugars simultaneously to ethanol are achieved where the XR, XD and XK genes are fused to promoters which are not inhibited by the presence of glucose and also do not require xylose for induction.

Another preferred embodiment of the invention provides a recombinant yeast strain containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase, wherein said genes are fused to non-glucose-inhibited promoters and wherein said yeast is capable of fermenting xylose to ethanol. The recombinant yeast strain is preferably also capable of fermenting glucose to ethanol.

Other preferred embodiments of the invention relate to reagents useful for the production of recombinant

yeasts of the invention. Thus, the present invention also provides a recombinant DNA molecule comprising genes encoding xylose reductase, xylitol dehydrogenase, and xylulokinase. As well, the invention provides a vector comprising genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. In these reagents, the genes are preferably fused to promoters which are not inhibited by glucose and also do not require xylose for induction, so as to enable the expedient production of recombinant yeasts capable of simultaneously fermenting glucose and xylose to ethanol.

Another preferred embodiment of the present invention provides a method for obtaining a recombinant yeast capable of fermenting xylose to ethanol. This method includes the step of introducing DNA into a yeast so as to cause the yeast to have introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. Preferably, these genes will be fused to non-glucose-inhibited promoters to enable simultaneous fermentation of glucose and xylose to ethanol. Advantageously, all three genes can be introduced simultaneously, for instance using reagents of the invention as discussed above.

Still other preferred embodiments of the invention provide methods for fermenting xylose or glucose to ethanol. The inventive methods include the step of fermenting a xylose-containing or glucose-containing medium with a recombinant yeast strain containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase. It is desirable that the three introduced genes be fused to non-glucose-inhibited promoters, and that the medium contain both glucose and xylose, so as to provide the concurrent fermentation of

xylose and glucose to ethanol.

Additional preferred embodiments, features and advantages of the invention will be apparent from the following description.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is a schematic diagram of the enzymes associated with early stages of xylose metabolism in bacteria and yeasts.

5 Figure 2 shows the nucleotide sequence and deduced amino acid sequence of the yeast xylulokinase gene including its 5'- and 3'-flanking sequences. The initiation codon and stop codon are underlined. The possible control sequences in the 5' and 3' non-coding 10 regions are indicated by arrows.

Figure 3 shows the genes cloned on and the restriction map of the plasmid pLSK15.

Figure 4 shows the genes cloned on and the restriction map of the plasmid pUCKm10.

15 Figure 5 shows the genes cloned on and the restriction map of the plasmid pLNH21.

20 Figure 6A shows an HPLC chromatogram of a fermentation broth obtained by fermenting xylose with recombinant yeast SC (pLNH21) (*S. cerevisiae* containing introduced XR, XD and XK genes) for (I) 2 days; and (II) 4 days.

Figure 6B shows an HPLC chromatogram of a fermentation broth obtained by fermenting xylose with recombinant yeast SC (pLNH13-32) (*S. cerevisiae* containing introduced XR and XD but not XK genes) for (I) 2 days; and (II) 4 days.

Figure 6C shows an HPLC chromatogram of a

fermentation broth obtained by fermenting xylose with an un-engineered *S. cerevisiae* yeast (containing no introduced XR, XD or XK genes) for (I) 2 days; and (II) 7 days, as further described in Example 6.

5 Figure 7 shows the genes cloned on and the restriction map of plasmid pLNH33.

Figure 8A shows an HPLC chromatogram of a fermentation broth obtained by fermenting a glucose- and xylose-containing medium (10% and 5%, respectively) with un-engineered yeast strain 1400 (containing no introduced XR, XD or XK genes) for (I) 0 days; and (II) 2 days, as further described in Example 8.

15 Figure 8B shows an HPLC chromatogram of a fermentation broth obtained by fermenting a glucose- and xylose-containing medium (10% and 5%, respectively) with recombinant yeast 1400 (pLNH33) (yeast 1400 containing introduced XR, XD and XK genes) for (I) 0 days; and (II) 2 days, as further described in Example 8.

Figure 9 is a schematic diagram outlining the construction of pBluescript II KS(-) containing the cloned XR, XD, and XK genes: four such plasmids were constructed: pKS(-)-KK-A*R-KD-1; pKS(-)-KK-A*R-KD-2; pKS(-)-KK-AR-KD-3; and pKS(-)-KK-AR-KD-4, as further described in Example 4.

25 Figure 10 shows direct amplification of the intact xylitol dehydrogenase gene and the promoterless XD from *P. stipitis* chromosomal DNA by the polymerase chain reaction (PCR) technique; from left, Lane 1: Molecular markers (BamHI-digested DNA); Lane 2: *Pichia xylo-* dehydrogenase gene (intact); Lane 3: *Pichia xylitol*

dehydrogenase gene (promotorless); and Lane 4: Molecular markers, HaeIII digested ϕ X DNA.

Figure 11 diagrams the strategies used for sequencing the yeast xylulokinase gene.

- 5 Figure 12 is a schematic diagram outlining the construction of the plasmid pLNH21.

- Figure 13 shows an HPLC chromatogram of a fermentation broth obtained by fermenting a mixture of glucose (10%) and xylose (5%) with *S. cerevesiae* SC 10 (pLNH13-32) (containing only the XR and XD genes) for (I) 0 days; (II) 2 days; and (III) 5 days.

- Figure 14 shows an HPLC chromatogram of the fermentation broth obtained by fermenting a mixture of glucose (10%) and xylose (5%) with unengineered *Pichia* 15 *stipitis* for (I) 0 days; (II) 3 days; and (III) 5 days.

DETAILED DESCRIPTION

For the purposes of promoting an understanding of the principles of the invention, reference will now be made to certain embodiments thereof and specific language will be used to describe the same. It will nevertheless be understood that no limitation of the scope of the invention is thereby intended, such alterations, further modifications and applications of the principles of the invention as illustrated herein being contemplated as would normally occur to one skilled in the art to which the invention pertains.

The present invention provides recombinant yeasts, DNA molecules and vectors comprising XR, XD and XK genes. Such genes are well known to occur in a wide variety of microorganisms and, in fact, as discussed hereinabove, numerous XR, XD and XK genes have been identified and isolated. The particular source of these genes is not critical to the broad aspects of this invention; rather, any DNAs encoding proteins (enzymes) having xylose reductase activity (the ability to convert D-xylose to xylitol with NADPH or NADH as cofactor), xylitol dehydrogenase activity (the ability to convert xylitol to D-xylulose with NAD^+ as cofactor), or xylulokinase activity (the ability to convert D-xylulose to D-xylulose-5-phosphate) will be suitable. These genes may be obtained as naturally-occurring genes, or may be modified, for example, by the addition, substitution or deletion of bases to or of the naturally-occurring gene, so long as the encoded protein still has XR, XD or XK activity. Similarly, the genes or portions thereof may be synthetically produced by known techniques, again so long as they have the desired XR, XD or XK activity.

As examples, suitable sources of XR and XD genes include xylose-utilizing yeasts such as Candida shehatae, Pichia stipitis, Pachysolen tannophilus, suitable sources of XR genes include the above-noted xylose-utilizing yeasts, as well a xylose non-utilizing yeasts such as those from the genus Saccharomyces, e.g. S. cerevisiae, the genus Schizosaccharomyces, e.g. Schizosaccharomyces pombe, and bacteria such as Escherichia coli, Bacillus species, Streptomyces species, etc. Genes of interest can be recovered from these sources utilizing conventional methodologies. For example, hybridization, complementation or PCR techniques can be employed for this purpose.

The particular XR gene used in the applicants' studies herein was cloned from P. stipitis by Polymerase Chain Reaction (PCR) (Chen and Ho, 1993). The oligonucleotides required for the amplification of XR from the chromosomal DNA by PCR were synthesized according to the published sequence of the P. stipitis XR gene (Takama et al., 1991). The amplified XR was first cloned and stored into plasmid pUC19. The cloned XR was then fused to different promoters including the promoters of yeast TRP5 gene (Zalkin and Yanofsky, 1982) and yeast alcohol dehydrogenase I gene (ADC1) (Ammerer, 1983; Bennetzen and Hall, 1982).

The XD gene used in the applicants' studies was also cloned from P. stipitis by PCR. The oligonucleotides required for the amplification of XD from the Pichia chromosomal DNA were synthesized according to the published sequence of the Pichia XD gene (Köetter et al., 1990). The amplified XD was also first cloned and stored in pUC19. The gene was then subsequently fused to

glycolytic promoters of yeast pyruvate kinase gene (PYK) (Burke et al., 1983) and yeast glyceraldehyde 3 phosphodehydrogenase gene (GPD) (Holland and Holland, 1979).

5 The applicants have cloned three different XK genes, those from S. cerevisiae (Ho and Chang, 1989), P. tannophilus (Stavis et al., 1987) and E. coli and have found that all three genes can be effectively expressed in S. cerevisiae after fusion to a highly efficient yeast promoter. The cloned S. cerevisiae xylulokinase gene was used in the illustrative work set forth herein. To assist in properly fusing the yeast XK gene to a suitable promoter, the complete nucleotide sequence of the S. cerevisiae XK gene including its 5' and 3' non-coding sequence has been analyzed and is shown in Figure 2.

15 A wide variety of promoters will be suitable for use in the invention. Broadly speaking, yeast-compatible promoters capable of controlling transcription of the XR, XD or XK genes will be used. Such promoters are available from numerous known sources, including yeasts, bacteria, and other cell sources. Preferably, the promoters used in the invention will be efficient, non-glucose-inhibited promoters, which do not require xylose for induction. In this regard, an "efficient" promoter as used herein refers to a promoter which provides a high level of transcription of the fused gene. Promoters having these characteristics are also widely available, and their use in the present invention, given the teachings herein, will be within the purview of the ordinarily skilled artisan, as will be the fusion of the promoters to the XR, XD and XK genes, the cloning of the promoter/gene fusion products into suitable vectors and the use of such vectors to transform yeast. All of these manipulations can be

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performed using conventional genetic engineering techniques well known to the art and literature.

More particularly describing the applicant's illustrative work herein, the yeast xylulokinase gene, XK, has been fused to promoters from yeast alcohol dehydrogenase gene (ADC1), yeast pyruvate kinase gene (PYK), yeast TRP5-gene, etc. XK fused to the TRP-5 promoter was used to construct pLNH21 (Figure 5) and XK fused to the PYK promoter was used to construct pLNH33 (Figure 7).

The fusion of XR, XD, and XK to intact promoters from ADC1, PYK, GPD, etc., was carried out by cloning both the fragment containing the specific promoter and the structural gene of XR, XD, or XK on one of the Bluescript KS plasmids (Stratagene, La Jolla, CA), followed by the removal of the extra unwanted nucleotides by site-specific mutagenesis (Kunkel et al., 1987). The invention thus also provides several pBluescript II KS(-) (hereinafter pKS(-)) derivatives containing the cloned XD (fused to the pyruvate dehydrogenase promoter), XR (fused to the ADC1 promoter), and XK (fused to the pyruvate kinase promoter). These recombinant plasmids are designated as pKS(-) KD-AR (or A*R) -KK. Four such plasmids were constructed as outlined in Figure 9. These plasmids have similar but not identical structures. The XR, XD, and XK (or KD-AR (or A*R) -KK) cloned on these plasmids can be separated from the parent pKS(-) plasmid by a single XhoI restriction digestion.

The XR, XD, and XK genes fused to the proper promoters were then cloned on pLSF5 (Figure 3) or pUCKm10 (Figure 4). pLSK15, a derivative of pL210-14 (Stevie and Ho, 1985), is a low copy number plasmid with a copy number

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of approximately 10 in yeast (*S. cerevisiae*). It contains the yeast 2 μ replicon which enables the plasmid to be replicated autonomously in *S. cerevisiae* and closely related species. pLSK15 also contains the geneticin (kanamycin) resistance gene (Km^R) and ampicillin resistance gene (Ap^R and also amp^r) which serve as selection markers in *S. cerevisiae* and other yeasts. pLSK15 also contains the XK gene fused to the yeast TRP-5 promoter. Thus, XR and XD genes fused to proper 5' noncoding sequences containing suitable promoters were inserted into pLSK15 to demonstrate the effect of the resulting plasmids on yeast xylose fermentation. To compare the effect of the presence of different genes on yeast xylose fermentation, a plasmid containing only XR and XD was also used to transform *S. cerevisiae* and the resulting yeast used in comparative fermentations. Results of the fermentation of xylose by un-engineered *S. cerevisiae*, yeast containing the cloned XR, XD, and XK (SC(pLNH21)), and yeast containing the cloned XR and XD but not XK (SC(pLNH13-32)) genes are shown in Figure 6A, 6B, and 6C.

pUCKm10 (Figure 4) is a high copy-number plasmid (i.e. plasmid with a copy number of about 50 or more) with a copy number close to 100 in *S. cerevisiae*. pUCKm10 is a pUC9 derivative containing the identical 2 μ replicon, and the Km^R , and Ap^R genes present in pLSK15. These specific DNA fragments serve as the replicon and selection markers that enable the plasmid to be replicated autonomously in *S. cerevisiae* (and in related yeasts) and also enable the yeast transformants containing the plasmid to be distinguished from the untransformed host cells.

The applicants have constructed pJCKm10 based recombinant plasmids that contain the same XR, XD, and XK

fused to 5' proper noncoding sequences containing suitable promoters. These vectors are designed to be useful to transform all S. cerevisiae strains and strains of related species. No special mutants are required to act as the 5 recipient strains. Thus plasmids such as pLNH33 (Figure 7), as well as pLNH21 (Figure 5), can be used to transform industrial S. cerevisiae and other strains.

Yeast transformation with derivatives of either pLSK15 or pUCKm10 was carried out by electroporation 10 generally using the the procedure described by Becker and Guarente (1991). Authentic yeast transformants containing derivatives of either pLSK15 or pUCKm10 were isolated as further described below. Km^R present in the plasmids served as the primary selection marker which renders any 15 host cells obtaining one of these plasmids resistant to a much higher concentration of geneticin present in the medium. However, some yeast cells can be induced to become resistant to the same level of geneticin of the transformants containing the plasmid. Thus, not every 20 geneticin resistant colony is a true transformant. It has been reported that Ap^R can be expressed in S. cerevisiae but the latter is resistant to ampicillin without the presence of Ap^R . Thus, Ap^R cannot serve as a selection marker for yeast plasmid-mediated 25 transformation. Nevertheless, yeasts that contain the highly expressed Ap^R will produce sufficient penicillinase and make it possible to identify colonies containing such yeasts on special solid plates by the penicillinase test (Chevallier and Aigle, 1979). The 30 latter test has provided a technique to identify the true transformants of S. cerevisiae and other yeasts from the geneticin resistant colonies.

Yeast xylose (or xylos and glucose) fermentation was

carried out using the inventive recombinant yeasts under anaerobic conditions as described in Examples 6 through 9. The consumption of sugars (xylose and glucose) and the formation of ethanol and other products such as xylitol were followed during fermentation by taking samples and analyzing them by HPLC as further described in Example 6.

For example, pLNH21 (Figure 5) was used to transform S. cerevisiae. The resulting transformant containing pLNH21 is designated SC(pLNH21), and can ferment 5% xylose nearly totally to ethanol in two to four days as demonstrated in Figure 6A.

As an additional example, pLNH33 (Figure 7) was used to transform yeast strain 1400 which is closely related to S. cerevisiae and has high tolerance to alcohol and temperature (D'Amore et al., 1989; D'Amore, 1990). The resultant genetically engineered yeast, designated 1400(pLNH33), can ferment 10% glucose and 5% xylose totally to ethanol in two to four days, without requiring high cell densities, as shown in Figures 8A and 8B.

pLNH33 is a more effective plasmid than pLNH21 for xylose fermentation because it is a higher copy-number plasmid. Furthermore, the XK in pLNH33 is fused to a more efficient promoter than the XK in pLNH21. S. cerevisiae has also been transformed with pLNH33, designated SC(pLNH33). Although SC(pLNH33) is much more effective in fermenting xylose or mixtures of xylose and glucose than SC(pLNH21), 1400(pLNH33) was found to be more effective in fermenting mixtures of glucose and xylose than SC(pLNH33). Thus, individual strains also affect the efficiency of fermentation. Similar to S. cerevisiae, the unengineered strain 1400 cannot use or ferment xylose (alone or in a mixture of glucose and xylose) as shown in

Figure 8B.

Generally, the results of these fermentive tests demonstrate that it is necessary that the yeast contain three introduced genes, XR, XD, and XK which have been properly fused to suitable promoters (preferably efficient glycolytic or other promoters that are not subject to glucose inhibition, and do not require xylose for induction) and to coordinately express these genes to make the yeast capable of fermenting xylose to ethanol only, and not to other by-products such as xylitol.

The results further demonstrate the importance of cloning a xylulokinase gene (XK) in addition to XR and XD in order to make yeasts ferment xylose effectively, particularly to ferment both glucose and xylose simultaneously when they are present in the same medium, such as in the hydrolyzates of cellulosic biomass. Similar to XR and XD, the cloned XK is preferably fused to a suitable efficient glycolytic or other promoter that is not subject to glucose inhibition, and which further does not require xylose for induction.

Also, the applicants found that yeast containing just the cloned XR and XD can only ferment glucose but not xylose to ethanol when both these sugars are present in the culture medium together (see Figure 13). Moreover, the applicants' results demonstrate that it is necessary for any yeast, including those xylose fermenting yeasts such as *P. stipitis* and *C. shihatae* to contain XR, XD and XK, fused to promoters that are not inhibited by the presence of glucose and also not requiring the use of xylose for induction in order to be able to ferment both glucose and xylose to ethanol when both these sugars are present together in the culture medium. Figure 13

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demonstrates that S. cerevisiae and related species containing only the cloned XR and XD genes, fused to proper promoters, can only ferment glucose but not xylose to ethanol when both these sugars are present in the culture medium. Similarly, Figure 14 demonstrates that unengineered P. stipitis containing its original XR, XD, and XK can ferment xylose when the latter sugar is the sole carbon source of the medium (results not shown) but it cannot ferment xylose when both glucose and xylose are the carbon sources present in the same medium.

It will be understood that for those yeasts that contain low levels of xylulokinase activity, introducing the XK gene serves two purposes. One is to improve the level of the enzyme activity. High levels of XK activity are important for more advantageous yeast fermentation of xylose to ethanol as opposed to xylitol. The other is to place the gene under the control of an efficient promoter that will not be inhibited by the presence of glucose. It is well known that natural wild-type microorganisms including yeasts cannot use other sugars for growth and fermentation if glucose is present in the cultural medium. Glucose will inhibit the synthesis of the enzymes required for metabolizing other sugar molecules (the so called "glucose" effect). Thus promoters from genes for the synthesis of sugar molecule metabolizing enzymes excluding glucose will not be preferred since these will not provide simultaneous fermentation of the two abundant sugars. In addition, it was found in the applicants' work that cell growth is also a prerequisite for induction. Thus, promoters requiring xylose for induction are not preferred for the expression of XR, XD or XK.

For the purpose of promoting a further understanding of the present invention and its advantages, the following

Examples are provided. It will be understood that these Examples are illustrative, and not limiting, in nature.

EXAMPLE 1

Synthesizing the XR and XD genes by PCR.

5 The synthesis of the intact or promotorless XR by PCR has been previously described (Chen and Ho, 1993). For the synthesis of XD by PCR, three oligonucleotides according to the nucleotide sequence of XD (Kötter et al., 1990) were synthesized and are listed below:

10 Oligonucleotide I: pTCTAGACCACCCTAAGTCG
Oligonucleotide II: pCACAATTAATGA
Oligonucleotide III: pGGATCCACTATAGTCGAAG

Oligonucleotides I and II were used to synthesize the intact XD gene and oligonucleotides II and III were used to synthesize the promotorless XD as shown in Figure 10. The intact XD and the promotorless XD were first cloned in pKS(-) plasmid. The intact XR was then subcloned on pUCKm10 (Figure 4) and the resulting plasmid pUCKm10-XD, was used to transform S. cerevisiae by electroporation as described in Example 5. The yeast transformants were used to assay the xylitol dehydrogenase activity to demonstrate that the cloned gene is intact and can be expressed in S. cerevisiae.

EXAMPLE 2

25 Fusion of the promotorless XD gene to the yeast pyruvate kinase gene promotor

Fusion of the XD gene to P_{pk} was chosen to illustrate the precise fusion of yeast metabolizing genes to intact promoters by site-directed mutagenesis. These promoters are either glycolytic promoters or promoters

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that will not be inhibited by the presence of glucose in the culture medium and also will not require the presence of xylose for induction.

The promoter fragment of yeast pyruvate kinase from
5 -910 to +23 (Burke et al., 1983) was synthesized by PCR as
described in Example 1 for the synthesis of the XD gene.
Both the P_{PK} fragment and the promoterless XD were
subcloned on pKS(-) plasmid and the undesired nucleotides
between the P_{PK} and the intact XD structural gene were
10 removed by site-specific mutagenesis according to the
procedure of Kunkel (Kunkel, 1987). The resulting fused
gene contains -910 to -1 promoter fragments from the
pyruvate kinase gene and +1 to +1963 nucleotides from the
Pichia XD gene. The resulting pKS(-) plasmid containing
15 P_{PK} -XD (or KD) is designated pKS(-)-KD or pKD2.

EXAMPLE 3

Analysis of the nucleotide sequence of yeast xylulokinase gene

The cloning of a 7.0 kb yeast (*S. cerevisiae*) DNA
20 fragment that contains the yeast xylulokinase gene has
been previously reported (Ho and Chang, 1989). By
subcloning, the XK gene has been located on a 2.4 kb
fragment. The nucleotide sequence of the 2.4 kb fragment
has been analyzed. The 5' non-coding region contains 345
25 nucleotides, the translated region contains 2118
nucleotides, and the xylulokinase encoded by XK has 591
amino acids as shown in Figure 2. The strategy used for
sequencing the XK gene is shown in Figure 11.

EXAMPLE 4

Construction of intact ADHI promoter

30

Plasmid pMA56 (Ammerer, 1983) contains the yeast

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alcohol dehydrogenase I promoter (P_{ADC1}). The applicants have used this promoter to modify some of the genes in their work. For example, P_{ADC1} has been fused to XR, and the resulting gene has been designated

- 5 P_{ADC1} -XR or AR. Nevertheless, this P_{ADC1} is not intact and does not contain the -1 to -14 nucleotides of the intact ADC1 promoter (Bennetzen and Hall, 1982). The -1 to -14 region of a gene is usually very significant for controlling protein synthesis. Any gene fused to such a
- 10 promoter has to rely on its original genetic signal for controlling the synthesis of its protein product.

- In order to better control the expression of the gene fused to the ADC1 promoter, the applicants employed site-specific mutagenesis to add the missing nucleotides
- 15 (-1 to -14) to the ADC1 promoter cloned on pMA56. The new intact ADC1 promoter is designated P_{ADC1}^* . This promoter has been used to modify XR and the resulting gene is designated as P_{ADC1}^* -XR or A^{*}R.

EXAMPLE 5

- 20 ~~Construction of plasmid pLNH21~~
(also designated as pLSK15-KD-AR)
and transformation of *S. cerevisiae*
and 1400 with pLNH21

The construction of pLNH21 is outlined in Figure 12.

- 25 pLNH21 was used to transform *S. cerevisiae* and strain 1400 by electroporation under the following conditions. Fifty ml yeast cells, grown to early log phase (Klett Unit (KU) 130), were centrifuged to remove the medium, washed twice with cold water, once with cold 1 M sorbitol, and
- 30 resuspended in 200 μ l 1 M sorbitol. Sixty μ l of the cells were transferred into a 4 ml presterilized plastic tube (with cap) and to which 0.1 μ g to 1 μ g plasmid DNA was added. Fifty μ l of the resulting cells and

plasmid mixture were pipetted into a precooled gene pulser cuvette with a 0.2 cm electrode gap and the content in the cuvette was subjected to pulse by the gene pulser with a pulse controller (BioRad) at 2.0 KV, 25 μ F, 200 ohms.

- 5 Immediately, .50 ml YEPD (1% yeast extract, 2% peptone, and 2% glucose) was added to the cuvette. The content of the cuvette was transferred to a new 4 ml sterilized plastic tube and incubated at 30°C for 1 hr.
- 10 YEPD and 50 μ g/ml G418 (geneticin). Fast growing colonies were selected and replicated on agar plates containing the same medium. The selected colonies were subjected to the ampicillin test (Chevallier and Aigle, 1979) until a positive one was identified. The
- 15 above-described electroporation procedure is based on that reported by Becker and Guarente (1971). Our method for the selection of G418 resistant transformants is very effective and most of the selected colonies that were replicated on plates containing YEPD plus 50 μ g/ml G418
- 20 were positive for the penicillinase test.

Transformation of strain 1406 with pLNH21 or other plasmids was carried out using a similar procedure to that described above, except that the cells were grown to 140-190 KU rather than 130 KU and the YEPD plates for the

25 initial selection of transformants after electroporation contained 40 μ g/ml geneticin G418 rather than 50. Transformation of strain 1400 by the above described procedures was not as effective as transformation of S. cerevisiae.

EXAMPLE 6

30

Example 6: A set of yeast with the following plasmids: SC(pLNH21), SC(pLNH13-32), and un-engineered parent S. cerevisiae

These three yeasts were cultured in rich medium YEPD aerobically under identical conditions (SC(pLNH13-32) was constructed by transforming S. cerevisiae with a plasmid, designated pLNH13-32, which contains only the XR and XD gene/promotor combinations). These yeast cells were then used to ferment 5% xylose in YEP (1% yeast extract, 2% ~~peptone~~) ~~medium anaerobically also under identical~~ conditions. The consumption of xylose and the formation of ethanol and xylitol were followed during fermentation by taking samples at proper intervals and analyzing them by HPLC under the following conditions.

The samples containing the fermentation broth (0.6 ml to 1.0 ml) removed from the cultures were kept in 1.5 ml Eppendorf tubes. The cells and other residues were first removed by centrifugation. The supernatant was further filtered by using sterile aerodisc (Gelman Sciences), 0.2 or 0.45 mm, syringe filters. The resulting filtrate from each sample was analyzed for its ethanol, glucose, xylose, and xylitol contents by high performance liquid chromatography (HPLC), using a Hitachi system according to the following conditions.

°Column: Aminex HPX-87C, 300 X 7.8 mm
°Mobile phase: water
°Flow rate: 0.8 ml/min.
°Detection: Hitachi L-3350 RI detector
°Temperature: 80°C
°Injection volume: 20 µl

The results, shown in Figures 6A, 6B, and 6C (ethanol smaller than they should be due to the sensitivity of the instrument), demonstrate that only the engineered yeast SC(pLNH21) containing the cloned XR, XD, and XK can

ferment high concentrations of xylose (5%) to ethanol, not the un-engineered parent *S. cerevisiae*, and also not the engineered SC(pLNH13-32) which only contains the cloned XD and XR, not XK. SC(pLNH13-32) ferments xylose mostly to 5 xylitol.

EXAMPLE 8
Effective fermentation of high concentrations of both glucose and xylose by 1400(pLNH33) to ethanol

10 A mixture of glucose and xylose (approximately 10% glucose and 5% xylose) were fermented by strain 1400 and 1400(pLNH33) under identical conditions. These yeasts were kept on agar plates containing the proper media and were inoculated directly from the agar plates into 50 ml 15 of YEPD medium (1% Yeast extract, 2% peptone, and 2% glucose) in a 250 ml Erlenmeyer flask equipped with a side-arm which allows direct monitoring of the growth of the yeast cultures by the Klett colorimeter. The cultures were incubated in a shaker at 30°C and 200 rpm aerobically.

20 When the cell density reached mid-log phase (400 Klett units), 12.5 ml (40%) glucose and 6.25 ml (40%) xylose were added to each flask. After thorough mixing, 1 ml of the culture mixture was removed from the flask to serve as the zero sample. The flask was then sealed with 25 Saran wrap to allow fermentation to be carried out anaerobically. One ml samples of the fermentation broth (with some cells) were removed at proper intervals (every 24 hr.) to serve as samples for measuring the sugar and ethanol contents of the broth during fermentation. The 30 samples were analyzed by HPLC as described in Example 6. The results, shown in Figures 8A and 8B, demonstrate that the genetically engineered yeast 1400(pLNH33) can ferment 10% glucose and 5% xylose to ethanol simultaneously in two

to four days without requiring high cell density. On the other hand, the parent strain 1400 can only convert glucose to ethanol but not xylose. The fermentation was carried out under normal conditions, without requiring special medium, special pH, and also without requiring growth of yeast to high cell density. Thus the genetically engineered 1400(pLNH33) containing the XR, XD, and XK, all fused to glycolytic promoters and cloned on a high copy-number plasmid pUCKm10, can ferment high concentrations of both glucose and xylose simultaneously to ethanol in two to four days with very little xylitol produced as a by-product.

EXAMPLE 9
Attempted Fermentation of xylose/glucose
with engineered SC(pLNH13-32)

15

The fermentation procedure of Example 8 was repeated except using S. cerevisiae SC (pLNH13-32) (containing only the XR and XD genes) as the fermentive organism. The results, shown in Figure 13, demonstrate that such a genetic unengineered yeast containing only the XR and XD genes can ferment glucose but not xylose when both of these sugars are present in the fermented medium.

EXAMPLE 10
Attempted Fermentation of xylose/glucose
with unengineered Pichia stipitis

25

The fermentation procedure of Example 8 was repeated, except using unengineered Pichia stipitis as the fermentive organism. Samples of the fermentation broth were analyzed by HPLC after fermentation for (I) 0 day; (II) 2 days; and (III) 5 days. The results, shown in Figure 14, demonstrate that Pichia stipitis can ferment glucose, but not xylose when both of these sugars are

30

present in the same medium.

While the invention has been illustrated and described in detail in the drawings and foregoing description, the same is to be considered as illustrative and not restrictive in character, it being understood that 5 only the preferred embodiment has been shown and described and that all changes and modifications that come within the spirit of the invention are desired to be protected.

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5 incorporated herein by reference.

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- 25 (vii) Prior Application Data: None
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(2) Information for Seq ID No:1:

- 35 (i) Sequence Characteristics
 (A) Length: 2467 base pairs
 (B) Type: Nucleotide Amino Acid
 (C) Strandedness: Double
 (D) Topology: Linear
 (ii) Molecule Type: Genomic DNA

40 (xi) Sequence Description: SEQ ID NO:1:

GGATCCAAGA	CCATTATTCC	ATCAGAATGG	AAAAAAGTTT	AAAAGATCAC	50
GGAGATTTTG	TTCTTCTGAG	CTTCTGCTGT	CCTTGAAAAC	AAATTATTCC	100
GCTGGCCGCC	CCAAACAAAA	ACAACCCCGA	TTTAATAACA	TTGTCAAGT	150
ATTAGAAATT	TTCTTTTAC	AAATTACCAT	TTCCAGCTTA	CTACTTCCTA	200
TAATCCTCAA	TCTTCAGCAA	GCGACGCAGG	GAATAGCCGC	TGAGGTGCAT	250
AACTGTCACT	TTTCAATTCT	GCCAATGCAA	TCTCAGGCGG	ACGAATAAGG	300
GGGCCCTCTC	GAGAAAAACA	AAAGGAGGAT	GAGATTAGTA	CTTTAATGTTG	351

Met Leu
1

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	TGT	TCA	GTA	ATT	CAC	AGA	CAG	ACA	AGA	GAG	GTT	TCC	AAC	ACA	393
	Cys	Ser	Val	Ile	Gln	Arg	Gln	Thr	Arg	Glu	Val	Ser	Asn	Thr	
			5					10					15		
	ATG	TCT	TTA	GAC	TCA	TAC	TAT	CTT	GGG	TTT	GAT	CTT	TCG	ACC	435
5	Met	Ser	Leu	Asp	Ser	Tyr	Tyr	Leu	Gly	Phe	Asp	Leu	Ser	Thr	
			20					25					30		
	CAA	CAA	CTG	AAA	TGT	CTC	GCC	ATT	AAC	CAG	GAC	CTA	AAA	ATT	477
	Gln	Gln	Leu	Lys	Cys	Leu	Ala	Ile	Asn	Gln	Asp	Leu	Lys	Ile	
				35					40						
10	GTC	CAT	TCA	GAA	ACA	GTG	GAA	TTT	GAA	AAG	GAT	CTT	CCG	CAT	519
	Val	His	Ser	Glu	Thr	Val	Glu	Phe	Glu	Lys	Asp	Leu	Pro	His	
	45					50					55				
	TAT	CAC	ACA	AAG	AAG	GGT	GTG	TAT	ATA	CAC	GGC	GAC	ACT	ATC	561
15	Tyr	His	Thr	Lys	Lys	Gly	Val	Tyr	Ile	His	Gly	Asp	Thr	Ile	
	60					65					70				
	GAA	TGT	CCC	GTA	GCC	ATG	TGG	TTA	GGG	GCT	CTA	GAT	CTG	GTT	603
	Glu	Cys	Pro	Val	Ala	Met	Trp	Leu	Gly	Ala	Leu	Asp	Leu	Val	
			75				80					85			
20	CTC	TCG	AAA	TAT	CGC	GAG	GCT	AAA	TTT	CCA	TTG	AAC	AAA	GTT	645
	Leu	Ser	Lys	Tyr	Arg	Glu	Ala	Lys	Phe	Pro	Leu	Asn	Lys	Val	
			90					95					100		
	ATG	GCC	GTC	TCA	GGG	TCC	TGC	CAG	CAG	CAC	GGG	TCT	GTC	TAC	687
	Met	Ala	Val	Ser	Gly	Ser	Cys	Gln	Gln	His	Gly	Ser	Val	Tyr	
					105					110					
25	TGG	TCC	TCC	CAA	GCC	GAA	TCT	CTG	TTA	GAG	CAA	TTG	AAT	AAG	729
	Trp	Ser	Ser	Gln	Ala	Glu	Ser	Leu	Leu	Glu	Gln	Leu	Asn	Lys	
	115					120					125				
	AAA	CCG	GAA	AAA	GAT	TTA	TTG	CAC	TAC	GTG	AGC	TCT	GTA	GCA	771
30	Lys	Pro	Glu	Lys	Asp	Leu	Leu	His	Tyr	Val	Ser	Ser	Val	Ala	
		130				135						140			
	TTT	GCA	AGG	CAA	ACC	GCC	CCC	AAT	TGG	CAA	GAC	CAC	AGT	ACT	813
	Phe	Ala	Arg	Gln	Thr	Ala	Pro	Asn	Trp	Gln	Asp	His	Ser	Thr	
			145				150					155			
	GCA	AAG	CAA	TGT	CAA	GAG	TTT	GAA	GAG	TGC	ATA	GGT	GGG	CCT	855
35	Ala	Lys	Gln	Cys	Gln	Glu	Phe	Glu	Glu	Cys	Ile	Gly	Gly	Pro	
			160					165					170		
	CAA	AAA	ATG	GCT	CAA	TTA	ACA	GGG	TCC	AGA	GCC	CAT	TTT	AGA	897
	Leu			Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	Leu	
					175					180					

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GAC GAG TTA AAC AAA GAA CGG GAA AAT AAT TAT GAG AAG ACT 1485
 Asp Glu Leu Asn Lys Glu Arg Glu Asn Asn Tyr Glu Lys Thr 400
 385

AAC GAT TGG ACT CTT TTT AAT CAA GCT GTG CTA GAT GAC TCA 1527
 5 Asn Asp Trp Thr Leu Phe Asn Gln Ala Val Leu Asp Asp Ser 410
 405

GAA AGT AGT GAA AAT GAA TTA GGT GTA TAT TTT CCT CTG GGG 1569
 Glu Ser Ser Glu Asn Glu Leu Gly Val Tyr Phe Pro Leu Gly 425
 415

10 GAG ATC GTT CCT AGC GTA AAA GCC ATA AAC AAA AGG GTT ATC 1611
 Glu Ile Val Pro Ser Val Lys Ala Ile Asn Lys Arg Val Ile 440
 435

TTC AAT CCA AAA ACG GGT ATG ATT GAA AGA GAG GTG GCC AAG 1653
 Phe Asn Pro Lys Thr Gly Met Ile Glu Arg Glu Val Ala Lys 455
 445

15 TTC AAA GAC AAG AGG CAC GAT GCC AAA AAT ATT GTA GAA TCA 1695
 Phe Lys Asp Lys Arg His Asp Ala Lys Asn Ile Val Glu Ser 470
 460

CAG GCT TTA AGT TGC AGG GTA AGA ATA TCT CCC CTG CTT TCG 1737
 20 Gln Ala Leu Ser Cys Arg Val Arg Ile Ser Pro Leu Leu Ser 480
 475

GAT TCA AAC GCA AGC TCA CAA CAG AGA CTG AAC GAA GAT ACA 1779
 Asp Ser Asn Ala Ser Gln Gln Arg Leu Asn Glu Asp Thr 495
 485

25 ATC GTG AAG TTT GAT TAC GAT GAA TCT CCG CTG CGG GAC TAC 1821
 Ile Val Lys Phe Asp Tyr Asp Glu Ser Pro Leu Arg Asp Tyr 510
 500

CTA AAT AAA AGG CCA GAA AGG ACT TTT TTT GTA GGT GGG GCT 1863
 Leu Asn Lys Arg Pro Glu Arg Thr Phe Phe Val Gly Gly Ala 525
 515

30 TCT AAA AAC GAT GCT ATT GTG AAG AAG TTT GCT CAA GTC ATT 1905
 Ser Lys Asn Asp Ala Ile Val Lys Lys Phe Ala Gln Val Ile 540
 530

GGT GCT ACA AAG GGT AAT TTT AGG CTA GAA ACA CCA AAC TCA 1947
 35 Gly Ala Thr Lys Gly Asn Phe Arg Leu Glu Thr Pro Asn Ser 550
 545

GGT GCC CTT GGT GGT TGT TAT AAG GCC AT TCG TCA TCG TTA 1965
 Ala Met Cys Gly Cys Lys Ala Me 565
 555

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TAT GAC TCT AAT AAA ATT GCA GTT CCT TTT GAT AAA TTT CTG 2031
 Tyr Asp Ser Asn Lys Ile Ala Val Pro Phe Asp Lys Phe Leu 580
 570
 AAT GAC AAT TTT CCA TGG CAT GTA ATG GAA AGC ATA TCC GAT 2073
 Asn Asp Asn Phe Pro Trp His Val Met Glu Ser Ile Ser Asp 595
 585
 GTG GAT AAT GAA AAT TGG ATC GCT ATA ATT CCA AGA TTG TCC 2115
 Val Asp Asn Glu Asn Trp Ile Ala Ile Ile Pro Arg Leu Ser 610
 600
 10 CCT TAAGCGAACT GGAAAAGACT CTCATCTAAA ATATGTTTGA ATAATTTATC 2168
 Pro
 ATGCCCTGAC AAGTACACAC AAACACAGAC ACATAATATA CATAATATA 2218
 TATATATCAC CGTTATTATG CGTGACATG ACAATGCCCT TGTATGTTTC 2268
 GTATACTGTA GCAAGTAGTC ATCATTITGT TCCCGTTTCG GAAAATGACA 2318
 15 AAAAGTAAAA TCAATAAATG AAGAGTAAAA AACAATTAT GAAAGGGTGA 2368
 GCGACCAGCA ACGAGAGAGA CAAATCAAAT TAGCGCTTTC CAGTGAGAAT 2418
 ATAAGAGAGC ATTGAAAGAG CTAGGTTATT GTTAAATCAT CTCGAGCTC 2467

What is claimed is:

1. A recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose to
5 ethanol.
2. ~~The recombinant yeast of claim 1 wherein the~~
yeast is also effective for fermenting glucose to ethanol.
3. The recombinant yeast of claim 2 wherein the yeast is of the genus *Saccharomyces*.
- 10 4. The recombinant yeast of claim 3 wherein said genes are fused to non-glucose-inhibited promoters and the yeast is effective for simultaneously fermenting glucose and xylose to ethanol.
5. A recombinant DNA molecule comprising genes
15 encoding xylose reductase, xylitol dehydrogenase and xylulokinase.
6. The recombinant DNA molecule of claim 5 wherein said genes are fused to non-glucose-inhibited promoters.
7. A vector effective for transforming yeast and
20 comprising genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.
8. The vector of claim 7 wherein said genes are fused to non-glucose-inhibited promoters.
9. A method for obtaining a recombinant yeast
25 effective for fermenting xylose to ethanol, comprising introducing DNA into a yeast so as to cause the yeast to

have introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

10. The method of claim 9 wherein said introduced DNA comprises genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase.

11. The method of claim 9 wherein said yeast is of the genus *Saccharomyces*.

12. A method for fermenting xylose to ethanol, comprising fermenting a xylose-containing medium with a recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose to ethanol.

13. The method of claim 10 wherein the medium also contains glucose and the yeast is also effective for fermenting said glucose to ethanol.

14. The method of claim 13 wherein the yeast is of the genus *Saccharomyces*.

15. The method of claim 14 wherein said genes are fused to non-glucose-inhibited promoters and the yeast is effective for simultaneously fermenting glucose and xylose to ethanol.

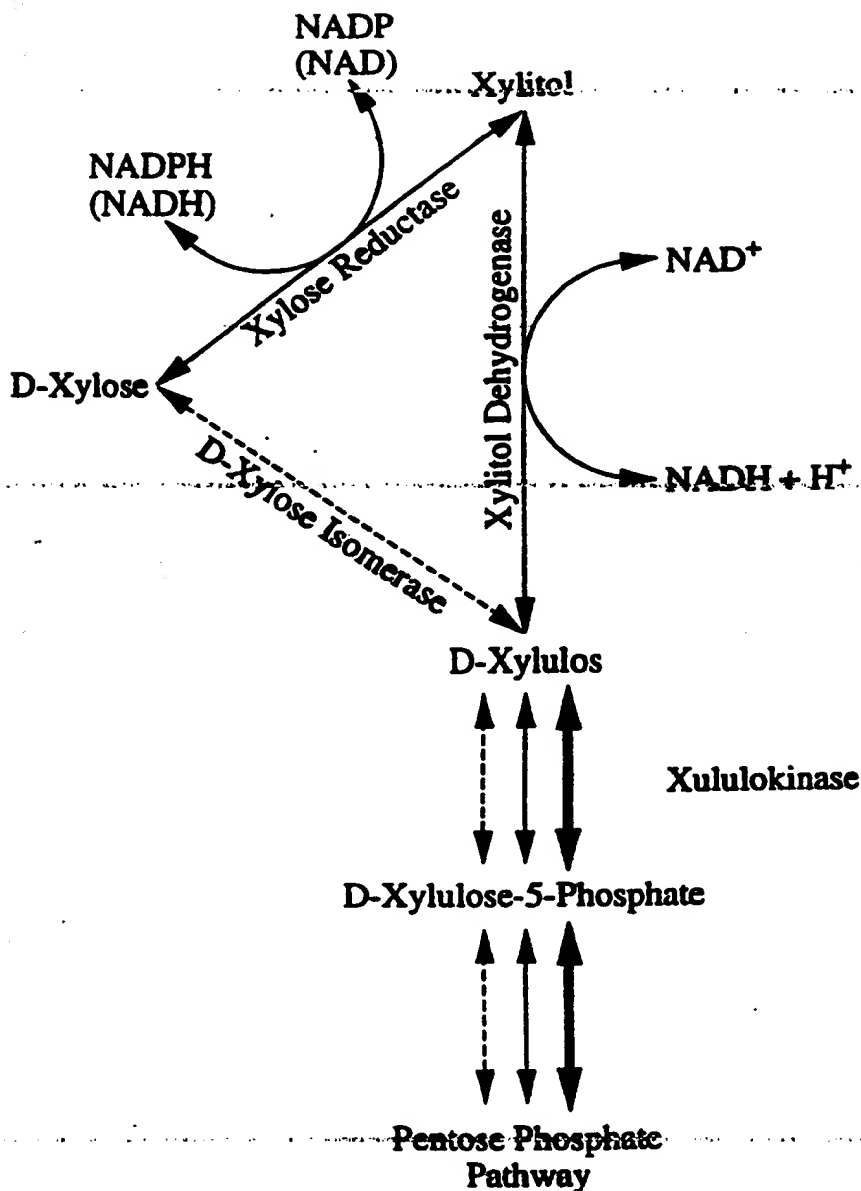
16. A method for fermenting glucose to ethanol, comprising fermenting a glucose-containing medium with a recombinant yeast containing introduced genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase and effective for fermenting xylose and glucose to ethanol.

17. The method of claim 16 wherein said medium also contains xylose.

18. The method of claim 17 wherein said yeast is of the genus *Saccharomyces*.

19. A recombinant yeast containing genes encoding xylose reductase, xylitol dehydrogenase and xylulokinase,
5 wherein said genes are fused to non-glucose-inhibited promoters and wherein said yeast is effective for fermenting xylose to ethanol.

20. The recombinant yeast of claim 19 wherein said yeast is also effective for fermenting glucose to ethanol.



The xylose metabolic pathways in microorganisms.

- Xylose non-utilizing yeasts (*Saccharomyces cerevisiae*, *Schizosaccharomyces pombe*, etc.)
- Xylose utilizing yeasts (*Candida shehatae*, *Pichia stipitis*, *Pachysolen tannophilus*, etc.)
- Bacteria (*E. coli*, *Bacillus* species, *Saccharomyces* species, etc.)

Figure 1.

WO 95/13362

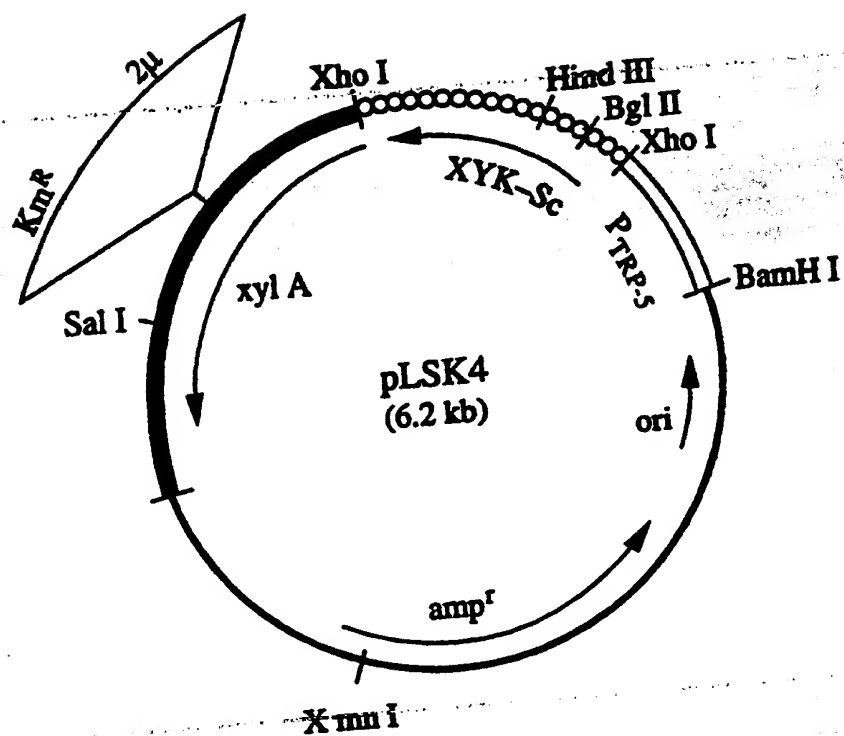
2/18

1 GGATCCAAGACCATTATTCCATCAGAATGGAAAAAGTTTAAAAGATCACGGAGATTTTG
61 TTCTTCTGAGCTTCTGCTGTCTTGAACAAATTATTCCGCTGGCCGCCCAACAAA
121 ACAACCCCGATTATAACATTGTCACAGTATTAGAAATTTCTTTTACAAATTACCAT
181 TTCCAGCTTACTACTTCCTATAATCCTCAATCTTCAGCAAGCGACGCAGGGAATAGCCG
241 TGAGGTGCATAACTGTCACTTTCAATTCCGCCAATGCAATCTCAGGCGGACGAATAAGG
301 GGGCCCTCTCGAGAAAAACAAAGGAGGATGAGATTAGTACTTTAATGTTGTGTTCA
361 ATTCAGAGACAGACAAGAGAGGTTTCCAACACAATGTCTTTAGACTCATACTATCTTGGG
421 TTTGATCTTTGACCCAACAACTGAAATGTCTCGCCATTAAACCAGGACCTAAAATTGTC
481 CATTGAGAAAGTGAATTTGAAAGGATCTCCGCATTATCACACAAAGAAGGGTGC
541 TATATACAGCGGACACTATCGAATGTCCCGTAGCCATGTGGTTAGGGCTCTAGATCTG
601 GTTCTCTGAAATATCGCGAGGCTAAATTTCCATTGAACAAAGTTATGGCCGTCTCAGG
661 TCCTGCCAGCAGCAGGGTGTCTACTGGTCTCCCAAGCCGAATCTCTGTTAGACAA
721 TTGAATAAGAAACCGAAAAAGATTATTGCACTACGTGAGCTCTGTAGCAATGCAAG
781 CAAACCGCCCAATTGGCAAGACCACAGTACTGCAAGCAATGTCAAGAGTTTGAAGAG
841 TGCATAGGTGGGCTGAAAAATGGCTCAATTAACAGGGTCCAGAGCCATTTAGATT
901 ACTGGTCTCAATTTCTGAAATTCACAATTAGAACCAGAAGCTTACGAAAAACAAG
961 ACCATTTCTTAGTGTCTAATTTTGTACTTCTATCTTAGTGGCCATCTTGTGAATTA
1021 GAGGAGGCAGATGCCTGTGGTATGAACCTTTATGATATACGTGAAAGAAATTCATGT
1081 GAGCTACTACATCTAATTGATAGTCTTCTAAGGATAAACTATCAGACAAAATTAATG
1141 AGAGCACCAGTAAAAATTTGATAGCGGGTACCATCTGTAATATTTTATTGAGAAGTAC
1201 GGTTCATACAACTGCAAGGTCTCTCCCATGACTGGGGATAATTTAGCCACTATATGT
1261 TCTTTACCCCTGCGGAAGAATGACGTTCTCGTTTCCCTAGGAACAAGTACTACAGTTCT
1321 CTGGTCACCGATAAGTATACCCCTCTCCGAATATCATCTTTTCAATCCAATCTG
1381 CCAACCATATATGGGTATGATTGTTAATGGTCTTTGGCAAGGGAGAGGATA
P N H Y M G M I C Y C N G S L A R E R I

Figure 2 (1/2)

1441 AGAGACGAGTTAAACAAAGAACGGGAAATAATTATGAGAAGACTAACGATTGGACTCTT
R D E L N K E R E N N Y E K T N D W T L
1501 TTTAATCAAGCAGTGCTAGATGACTCAGAAAGTAGTAAAAGAATTAGGTGTATATTTT
F N Q A V L D D S E S S E N E L G V Y F
1561 CCTCTGGGGGAGATCGTTCCTAGCGTAAAGCCATAAACAAAGGGTTATCTTCAATCCA
P L G E I V P S V K A I N K R V I F N P
1621 AAAACGGGTATGATTGAAAGAGAGGTGGCCAAAGTTCAAAGACAAGAGGCACGATGCCAAA
K T G M I E R E V A K F K D K R H D A K
1681 AATATTGTAGAATCACAGGCTTTAAGTTGCAGGGTAAGAATATCTCCCCTGCTTTCGGAT
N I V E S Q A L S C R V R I S P L L S D
1741 TCAAACGCAAGCTCACAACAGAGACTGAACGAAGATACAATCGTGAAGTTTGATTACGAT
S N A S S Q Q R L N E D T I V K F D Y D
1801 GARTCTCCGCTGCGGGACTACCTAAATAAAAGGCCAGAAAGGACTTTTTTTGTAGGTGGG
E S P L R D Y L N K R P E R T F F V G G
1861 GCTTCTAAAAACGATGCTATTGTGAAGAAGTTTGCTCAAGTCATTGGTGCTACAAAGGGT
A S K N D A I V K K F A Q V I G A T K G
1921 AATTTTAGGCTAGAAACACCAAACTCATGTGCCCTTGGTGGTTGTTATAAGGCCATGTGG
N F R L E T P N S C A L G G C Y K A M N
1981 TCATTGTTATATGACTCTAATAAAATTGCAGTTCCTTTTGATAAATTTCTGAATGACAAT
S L L Y D S N K I A V P F D K F L N D N
2041 TTTCCATGGCATGTAATGGAAAGCATATCCGATGTGGATAATGAAAATTGGATCGCTATA
F P W H V M E S I S D V D N E N W I A I
2101 ATTCCAAGATTGTCCCCTTAAGCGAACTGGAAAAGACTCTCATCTAAAATATGTTTGAAT
I P R L S P
2161 AATTTATCATGCCCTGACAAGTACACACAAACACAGACACATAATATACATACATATATA
2221 TATATCACCGTTATTATGCGTGACATGACAATGCCCTTGTATGTTTCGTATACTGTAGC
→ →
1281 AAGTAGTCATCATTTTGTTCCTCGGAAATGACAAAAAGTAAAATCAATAAATGAA
→
2341 GAGTAAAAACAATTTATGAAAGGGTGAGCGACCAGCAACGAGAGAGACAAATCAAATTA
2401 GCGCTTTCAGTGAGAATATAAGAGAGCATTGAAAGAGCTAGGTTATTGTTAAATCATCT
2461 CGAGCTC

Figure 2 (2/2)



pLSK15

Figure 3.

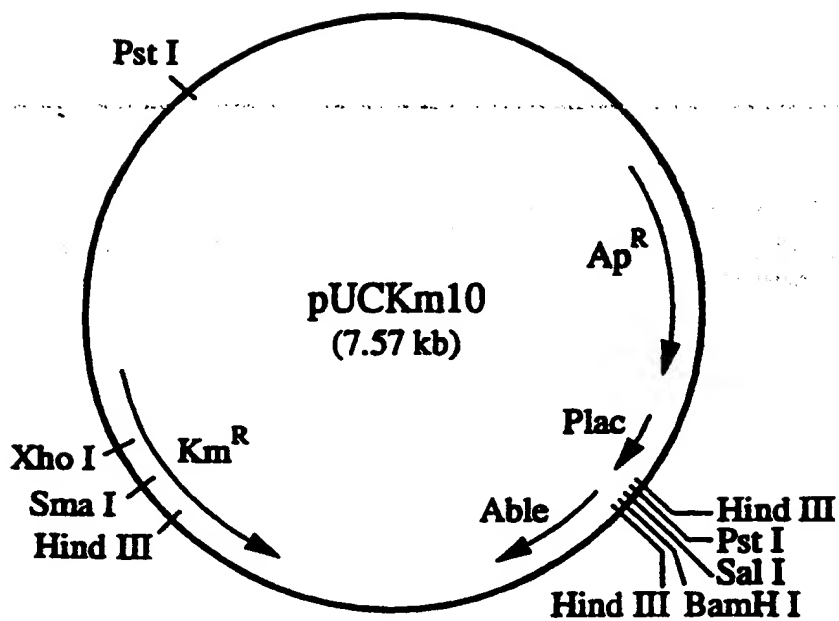
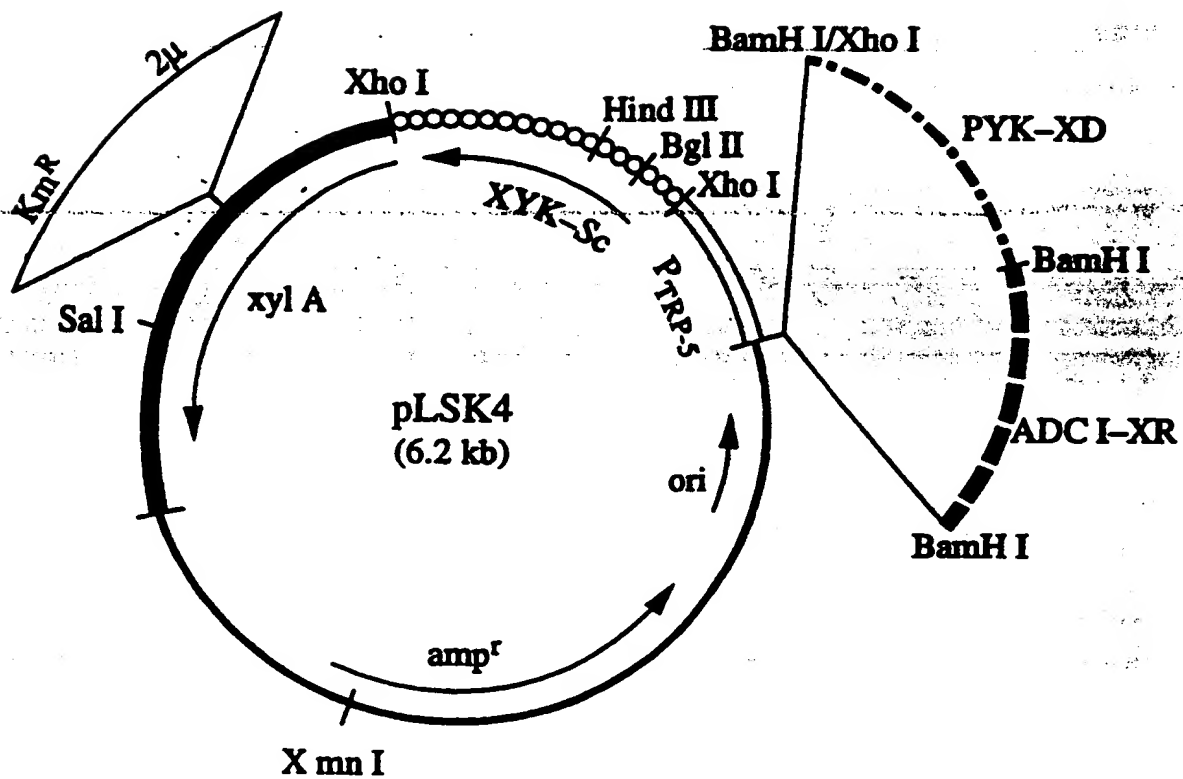


Figure 4.

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pLNH21

Figure 5.

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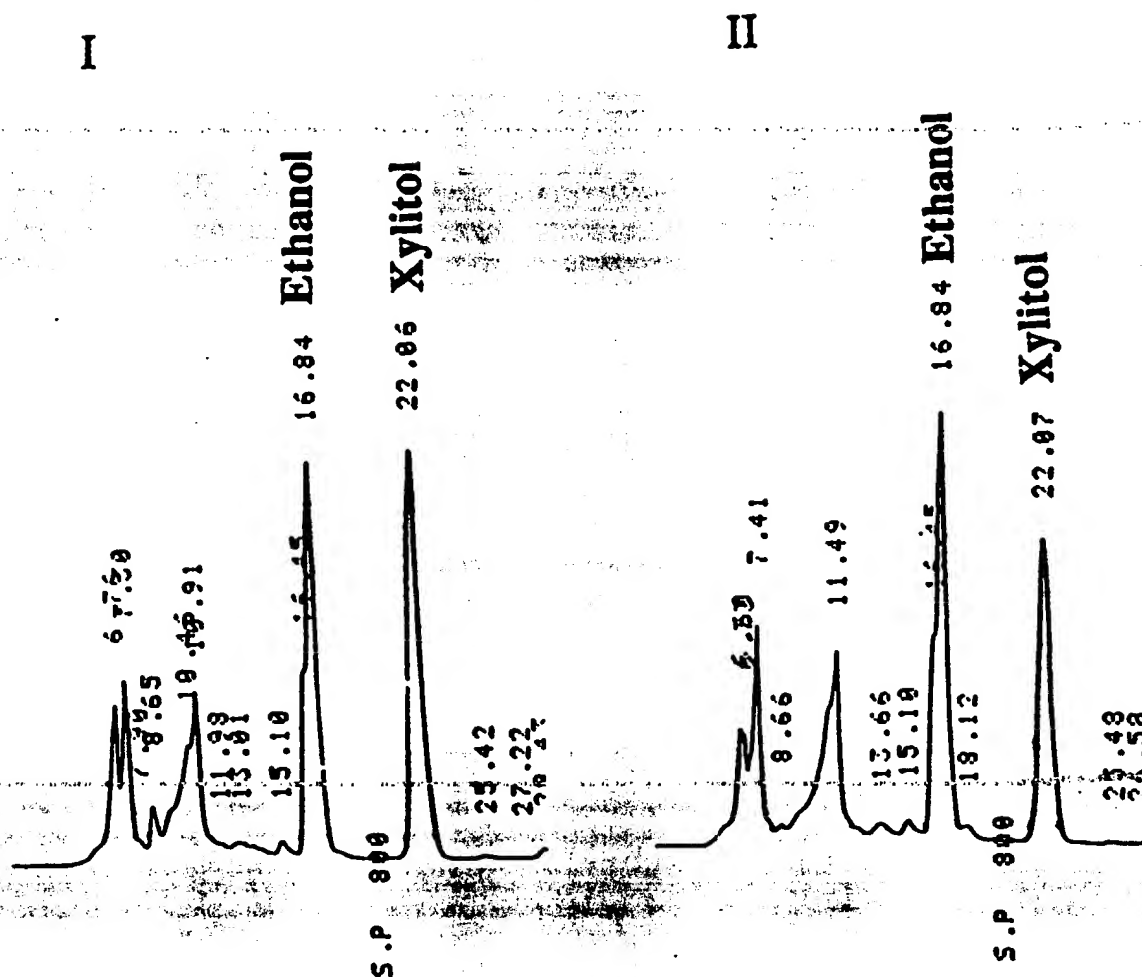


Figure 6A

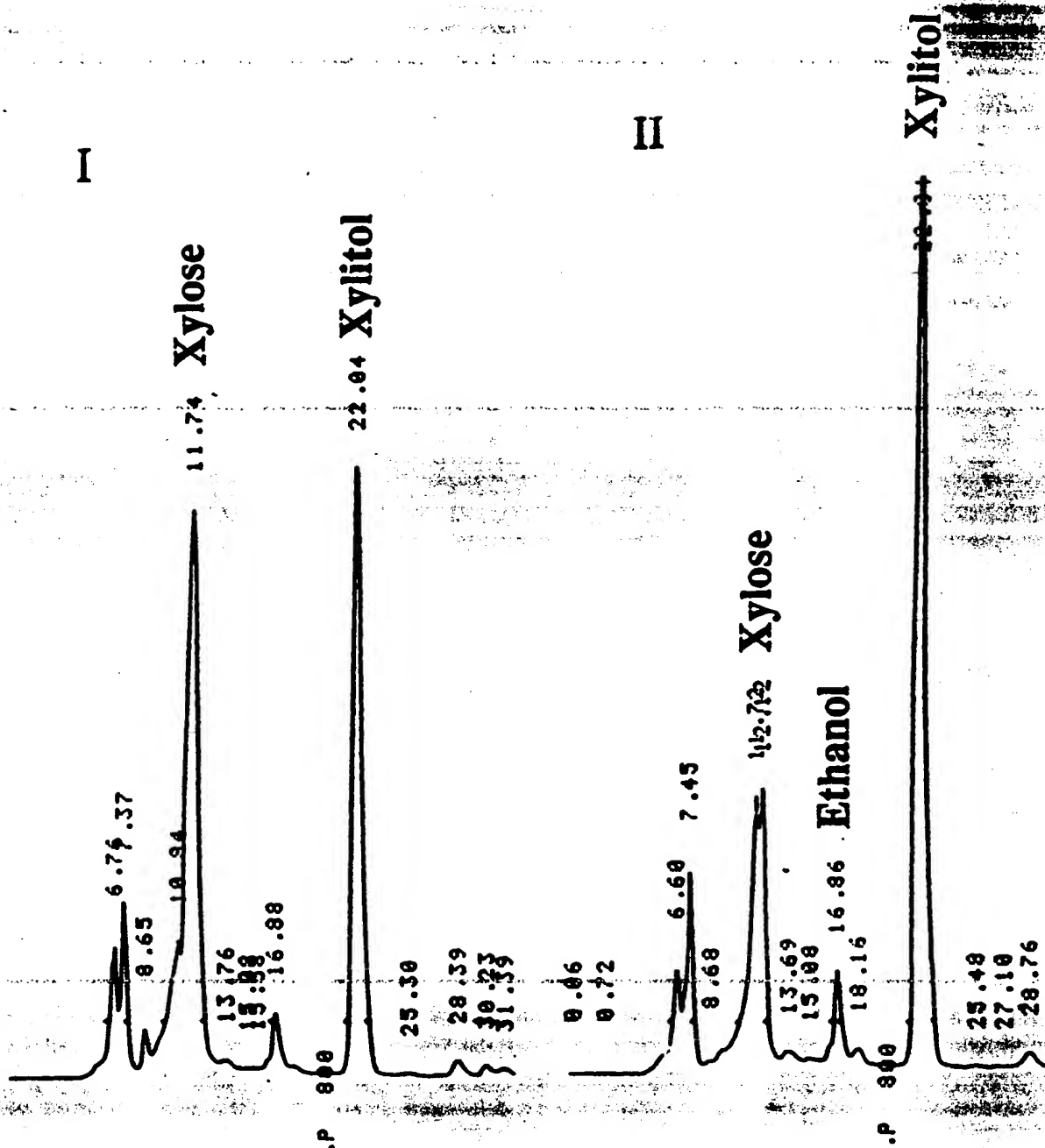


Figure 6B

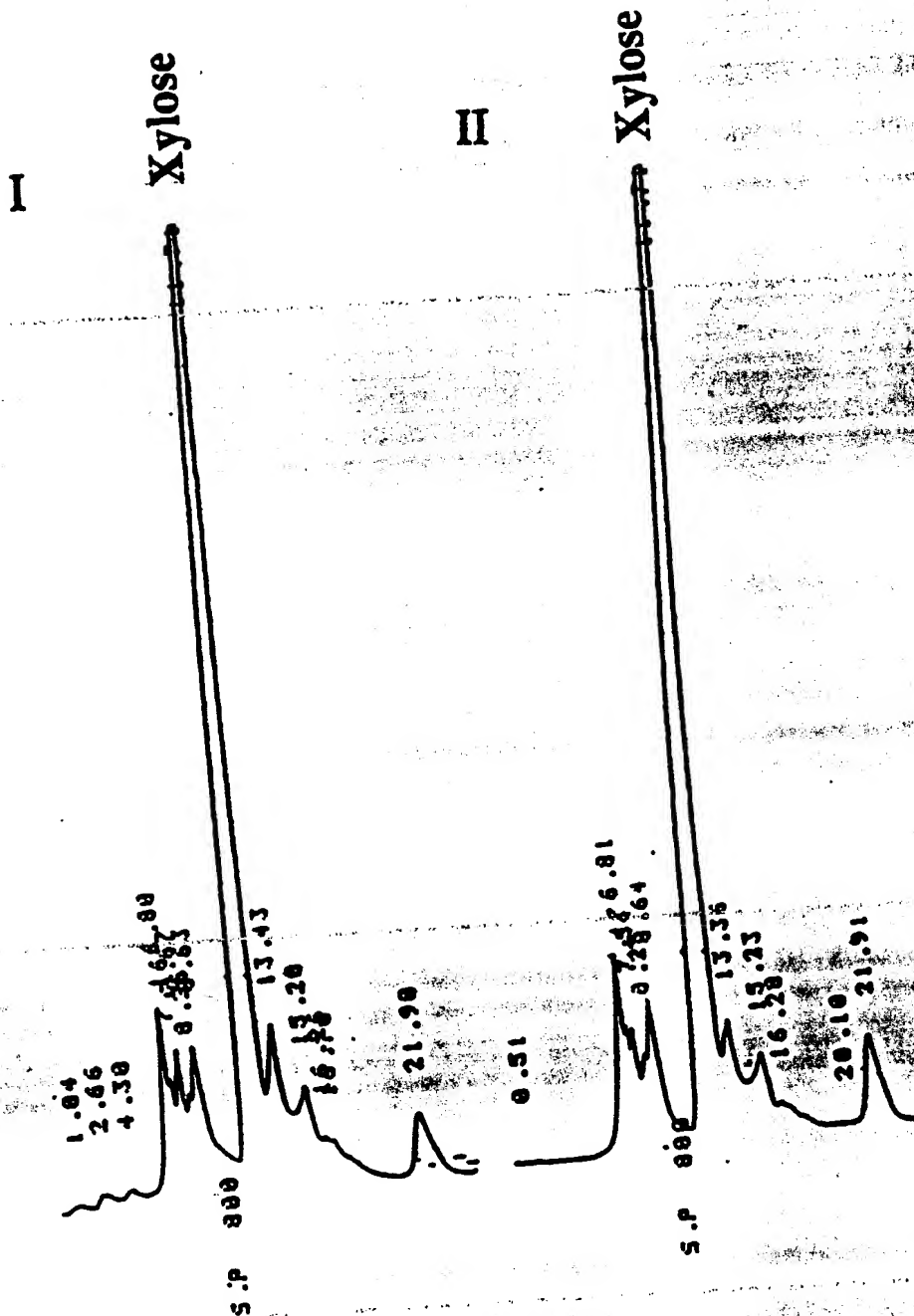


Figure 6C

10/18

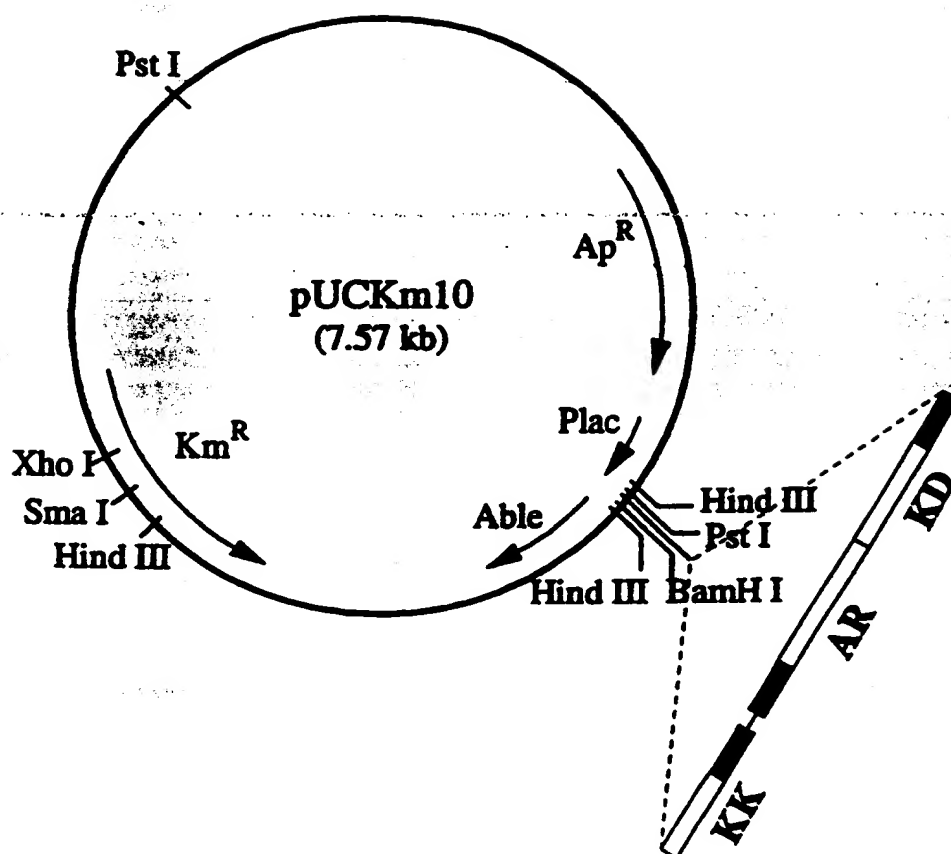
**pLNH33**

Figure 7.

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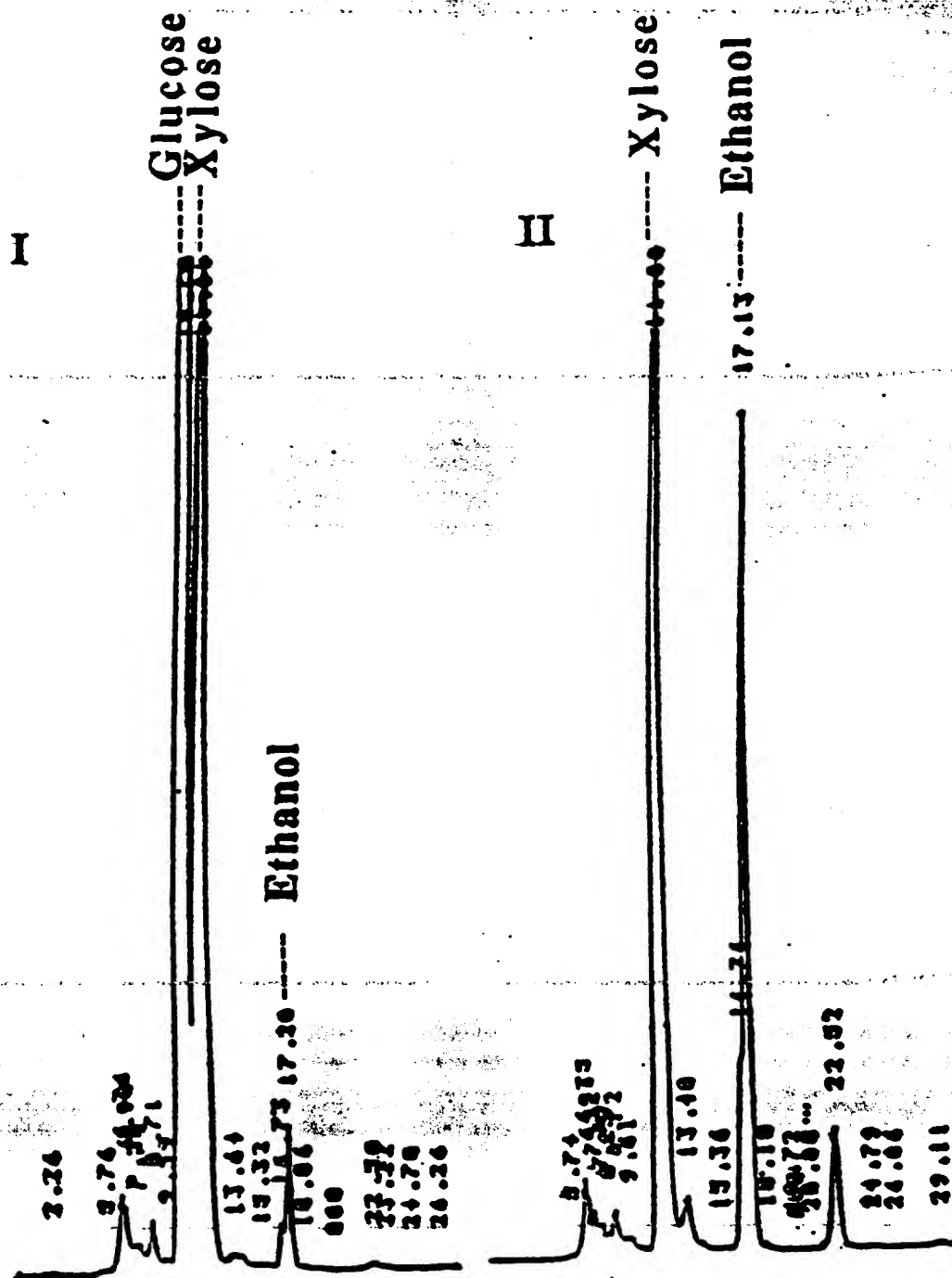


Figure 1

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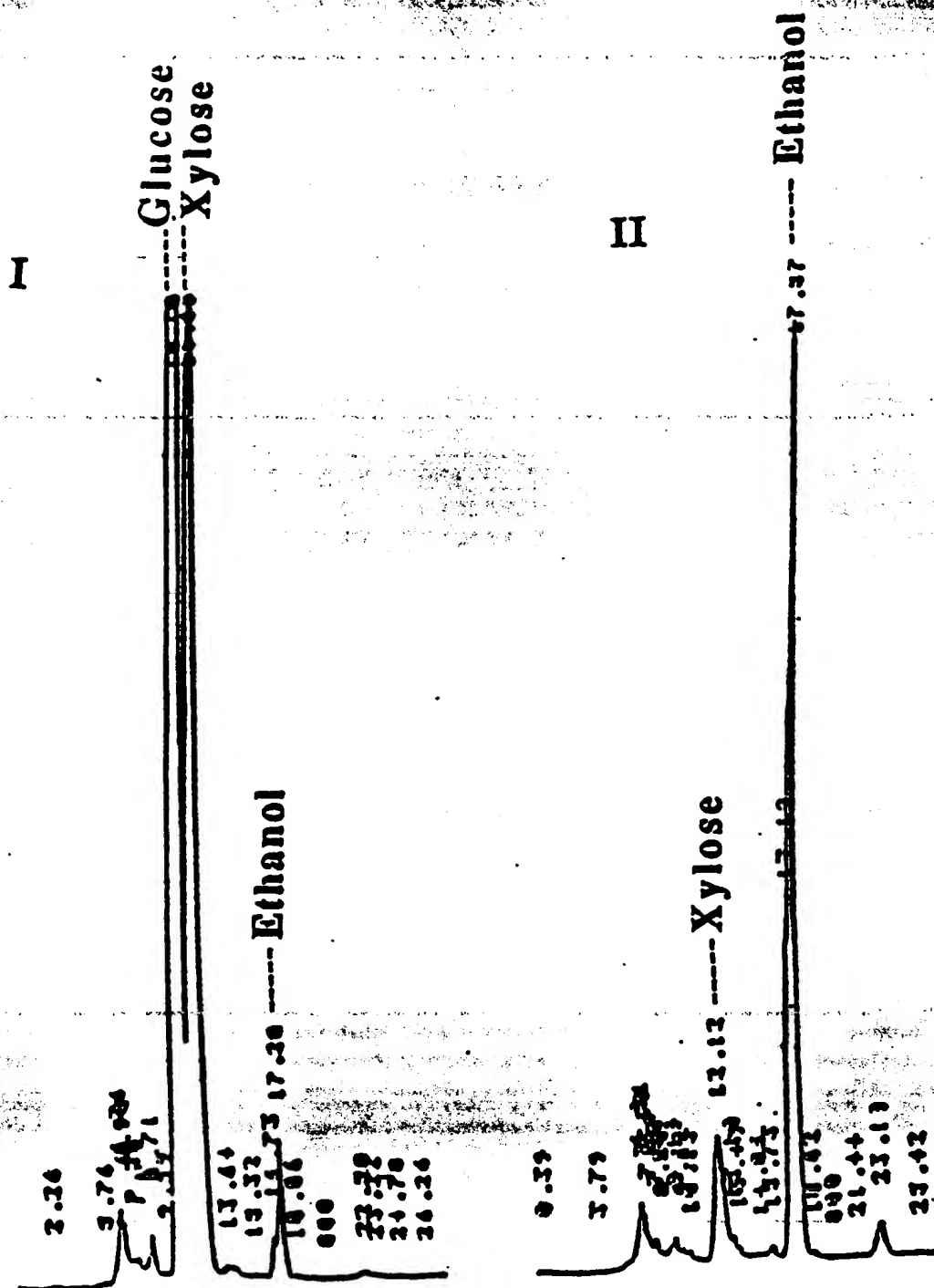


Figure 8B

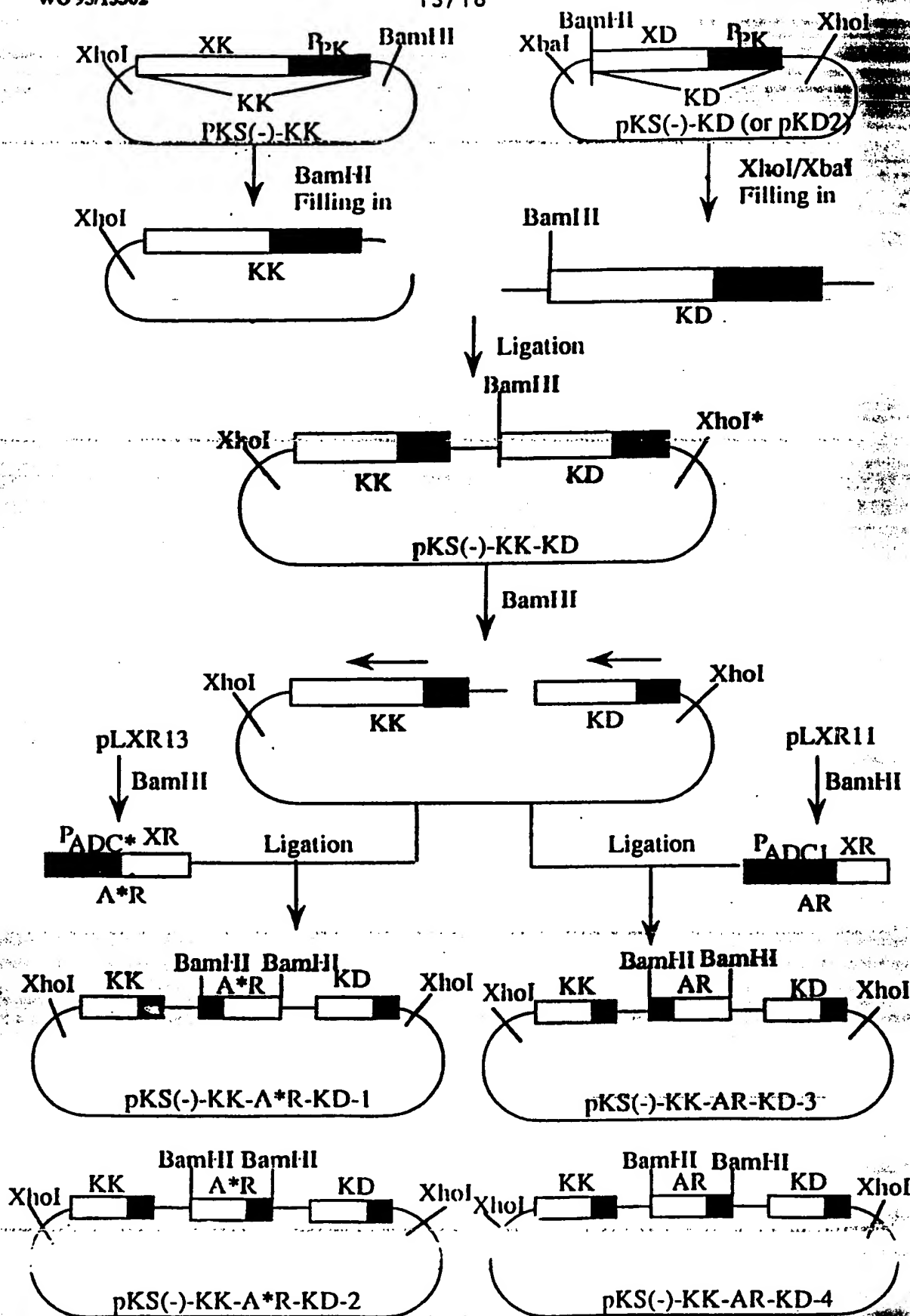


Figure 9. Construction of pKS(-)-KK-AR-KD plasmids

*The *XhoI* site was regenerated after ligation.

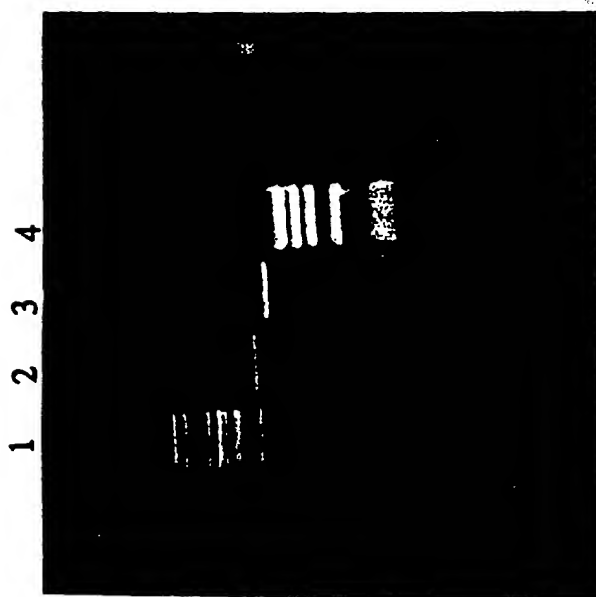


Figure 10.

Direct amplification of the intact xylitol dehydrogenase gene (XD) and the promoterless XD from *Pichia stipitis* chromosomal DNA by polymerase chain reaction (PCR) technique.

1. Molecular markers BamHI-EcoRI digested λ DNA.
2. *Pichia* xylitol dehydrogenase gene (intact).
3. *Pichia* xylitol dehydrogenase gene (promoterless).
4. Molecular markers, Hae III digested ϕ X-DNA.

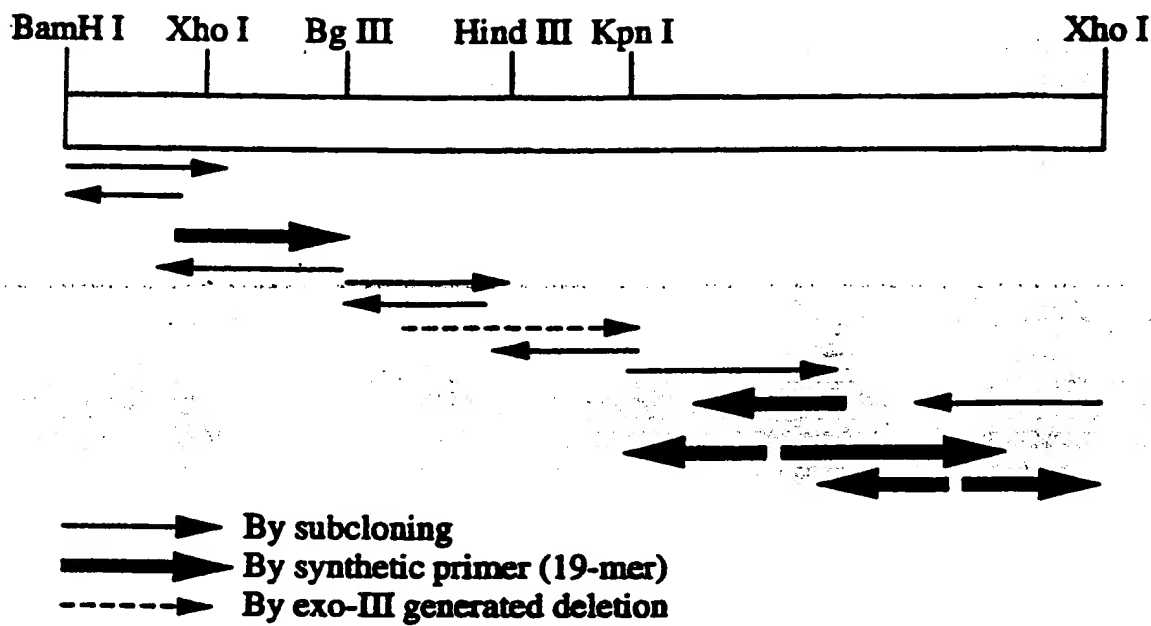


Figure 11.

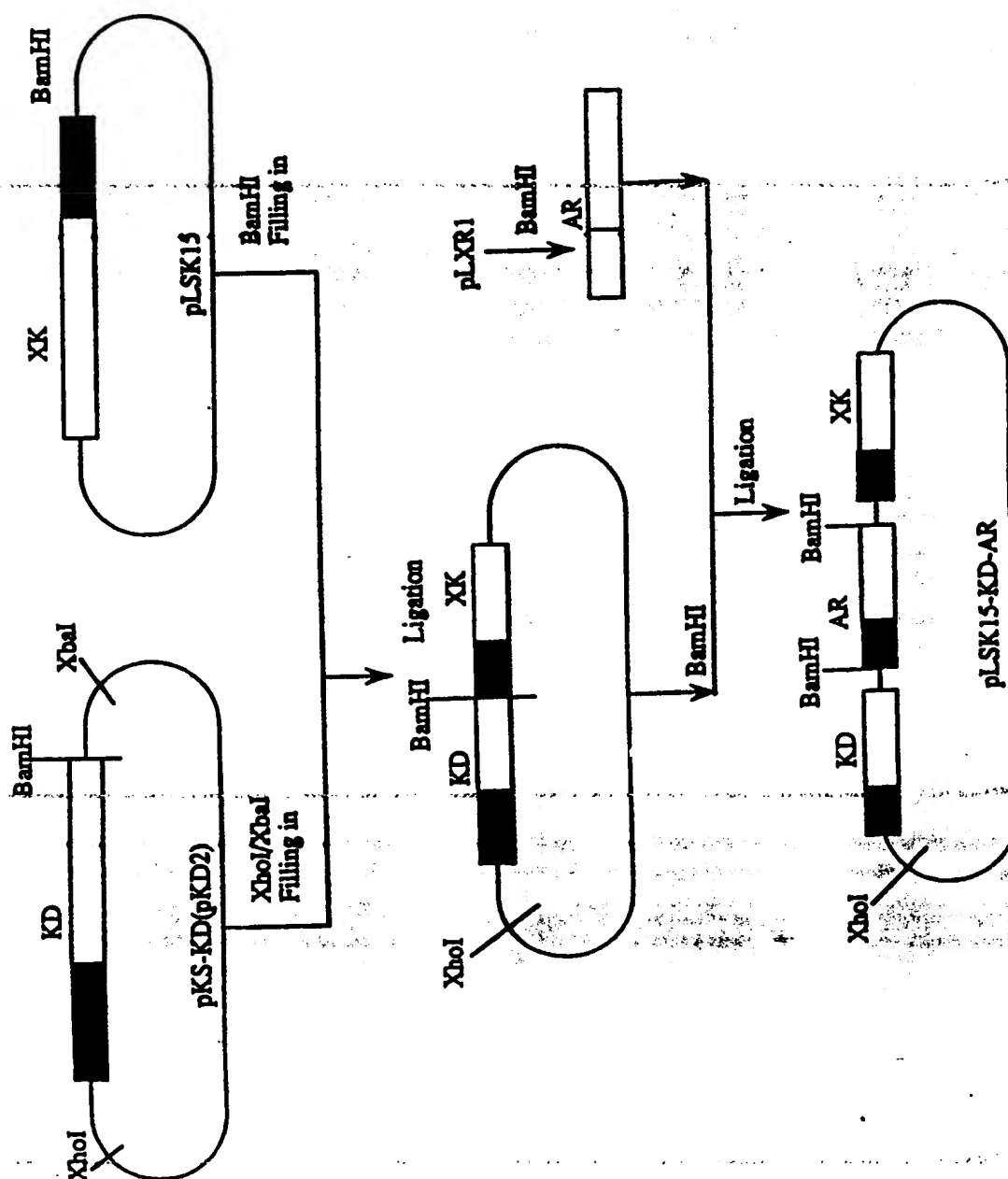


Figure 12

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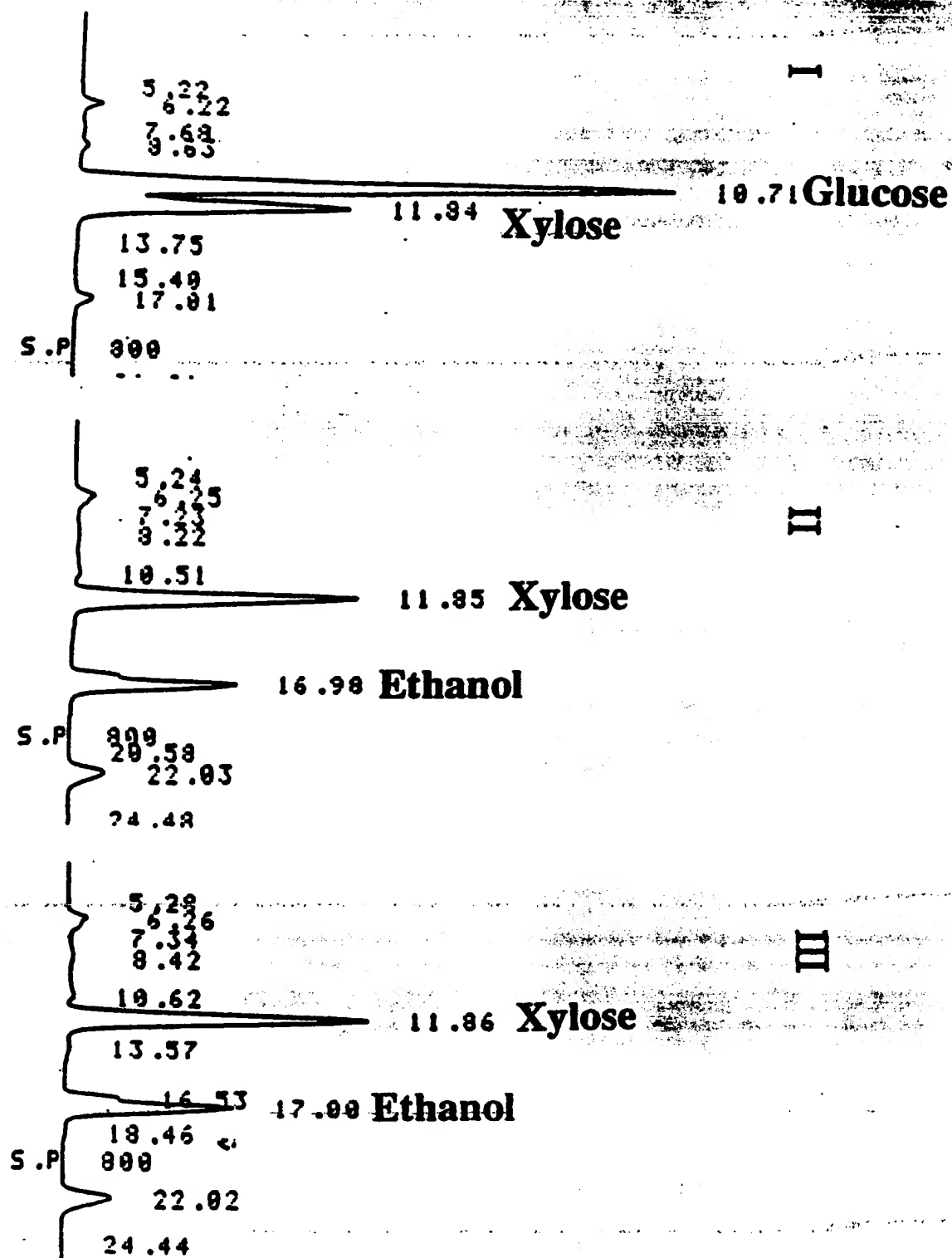


Figure 13

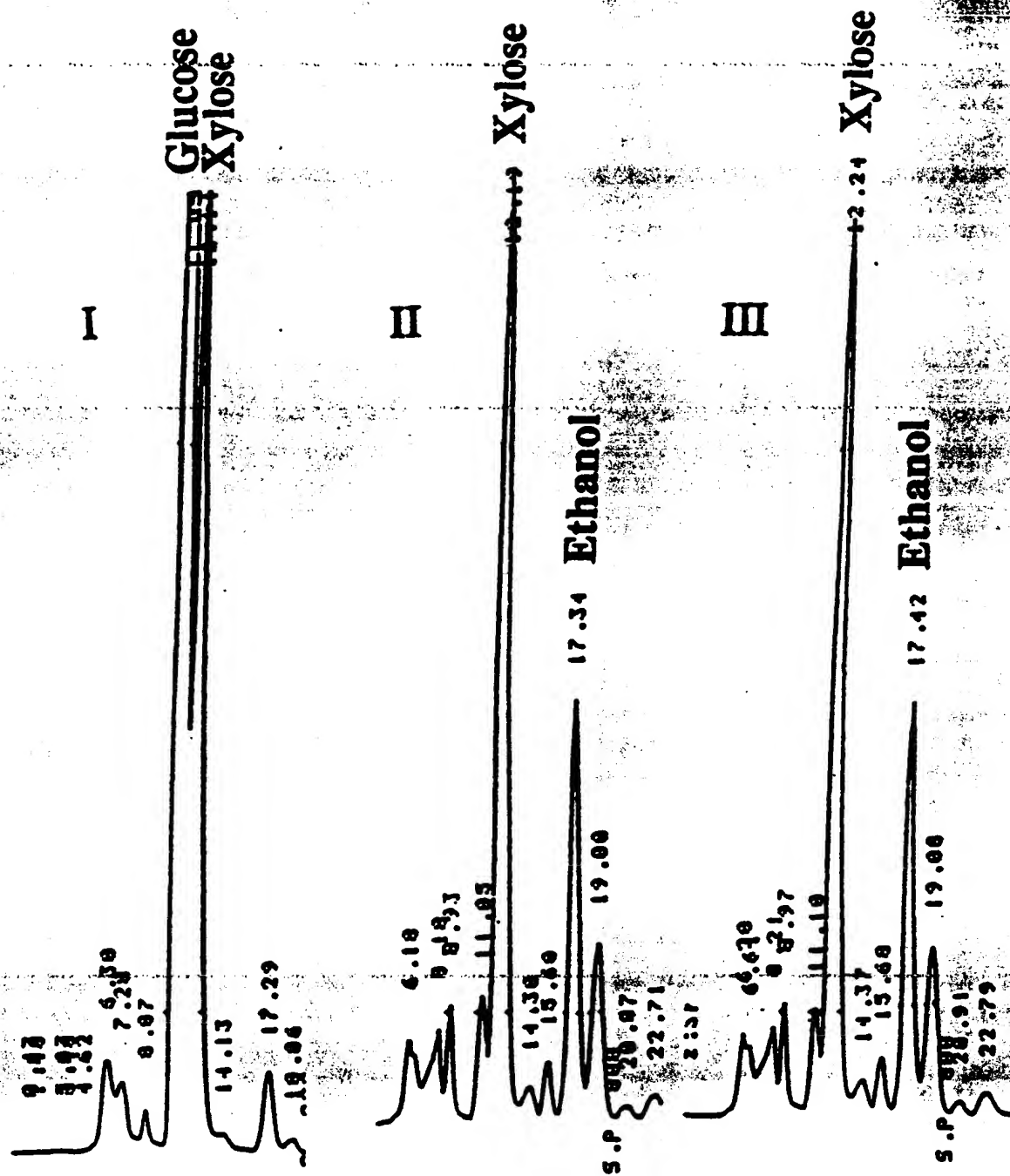


Figure 14

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US94/12861

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) : C12N 1/14, 9/00, 9/12, 15/00; C12P 7/08

US CL : 435/163, 172.3, 183, 194, 254.21, 320.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/163, 172.3, 183, 194, 254.21, 320.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, DIALOG

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	Applied Microbiology and Biotechnology, Volume 30, issued 1989, Amore et al., "The Fermentation of Xylose - an Analysis of the Expression of <i>Bacillus</i> and <i>Actinoplanes</i> Xylose Isomerase Genes in Yeast", pages 351-357, see entire document.	1-20
Y	The Journal of Biological Chemistry, Volume 258, Number 4, issued 25 February 1983, Burke et al., "The Isolation, Characterization, and Sequence of the Pyruvate Kinase Gene of <i>Saccharomyces cerevisiae</i> ", pages 2193-2201, see entire document.	2-20



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:	T	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	X	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier document published on or after the international filing date	Y	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)		
"O" document referring to an oral disclosure, use, exhibition or other means		
"P" document published prior to the international filing date but later than the priority date		

Date of completion of the international search

21 FEBRUARY 1995

Date of mailing of the international search report

24 FEB 1995

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Facsimile No. (703) 305-3230

Authorized officer

DOUGLAS GURIAN-SHERMAN

Telephone No. (703) 308-0196

PCT/US94/12261

Category*

Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

Y

Enzyme Microb. Technol. Volume 11, issued July 1989, Ho et al., "Cloning of Yeast Xylulokinase Gene by Complimentation of *E. coli* and Yeast Mutations", pages 417-421. see entire document.

1-20

Y

Current Genetics, Volume 18, issued 1990, "Isolation and Characterization of the *Pichia stipitis* Xylitol Dehydrogenase Gene, *XYL2*, and Construction of a Xylose-Utilizing *Saccharomyces cerevisiae* Transformant", pages 493-500, see entire document.

1-20